Insights into the regulation of human IgE : a complete picture in vitro

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To two wonderful parents.

INSIGHTS INTO THE REGULATION OF HUMAN IgE.

BY

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ABSTRACT.

In this project we investigated the effects of different CD8+ T-cell secreted factors on IgE switching. Human tonsillar B cells were isolated by T-cell rosetting or positive selection (CD19 dynabeads). B cells cultured with IL-4, anti-CD40 and holo-transferrin provided a good system in trigerring secretion of IgE "in vitro". IgE secretion within the first week of culture, produced levels that occasionally exceeded 2μg/ml. The IgE levels in the supernatants were tested by a direct, sandwich IgE ELISA, while B-cell proliferation assays were also carried out using ^{3}H-Thymidine. A wide range of cytokines were tested and their effects on B-cell proliferation and IgE secretion were confirmed using their respective neutralising antibodies. The effect of different CD8+ T-cell supernatants on the IL-4-dependent IgE synthesis was also tested. Finally the possible mode of action of these cytokines has been investigated.

Cytokines found to potentiate IgE secretion included IL-6 and IL-10, while IL-2, IL-12, TGF-β and IFN-γ downregulated the IL-4-dependent IgE production. The effect of these cytokines were confirmed by using their respective neutralising antibodies. Similarly, Tc clone supernatants were found to control the levels of IgE secreted in the B-cell cultures. Tc1 clone supernatants downregulated the secretion of IgE while the Tc2 clone supernatants increased the levels of this immunoglobulin.

The potentiating ability of IL-6 and IL-10 was probably due to their B-cell-proliferative effect while the inhibitory action of cytokines such as IFN-γ and TGF-β could be partially explained via the preferential switching theory; i.e. switching towards a different immunoglobulin isotype (e.g.: TGF-β to IgA and IFN-γ to IgG1) or due to the downregulation of the IL-4 receptor expression (seen with IL-12, IFN-γ and TGF-β).

ABBREVIATIONS.

µl	microlitres
AET	2-aminoethylisothiouronium bromide
APC	antigen presenting cell
BSA	bovine serum albumin
C	constant
CD	cluster of differentiation
cDNA	complementary deoxy-ribose nucleic acid
cpm	counts per minute
CTL	cytotoxic T lymphocytes
DNA	deoxy-ribose nucleic acid
DTH	delayed-type hypersensitivity
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence-activated cell sorter
FCS	foetal calf serum
FITC	fluorescein isothiocynate
g	gravitational force
h	hour
HIV	human immunodeficiency virus
IFN	interferon
Ig	immunoglobulin
IL	interleukin
LRP	linear regression plot

mAb	monoclonal antibody
MHC	major histocompatibility complex
ml	mililitre
mRNA	messenger ribose nucleic acid
ng	nanogram
NK	natural killer
OVA	ovalbumin
PBMC	peripheral blood nuclear cells
PBS	phosphate buffer solution
PCR	polymerase chain reaction
PE	phycoerythrin
R	receptor
RNA	ribose nucleic acid
RPMI	roswell park memorial institute
SD	standard deviation
SEM	standard error of mean
SRBC	sheep red blood cells
STAT	signal transducer and activator of transcription
SUP	supernatant
Tc	cytotoxic T cell
TcR	T-cell receptor
TGF	tumour growth factor
Th	helper T cell

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CHAPTER 1: INTRODUCTION

1.1 CELLS OF THE IMMUNE SYSTEM.

Pluripotent stem cells in the bone marrow give rise to all haematopoietic cells involved in the immune system (185,200) (Figure 1.1). Co-operation between the multiple cell types allows the immune system to identify the nature of antigen and mount a specific response, utilising the most efficient means of defence, as well as retaining specific memory.

1.2 CELLULAR AND HUMORAL IMMUNITY.

Cellular immunity (cell mediated) and humoral immunity (antibody mediated) work together during an immune response to antigen. The immune system is able to recognise the antigen, recruit appropriate cells and produce the most effective immune response to combat the invading pathogen. Cellular immunity involves cell mediated killing and clearance by leukocytes such as neutrophils, macrophages, natural killer (NK) cells, cytotoxic T (Tc) cells, eosinophils and basophils. Humoral immunity involves B cells (bone marrow derived lymphocytes) which secrete immunoglobulin (Ig) molecules (antibodies). Igs bind to antigen and can facilitate phagocytosis (opsonisation) of antibody targeted pathogens and complement mediated lysis via the activation of the classical pathway. Immunoglobulins also bind to their relevant Ig receptors on monocytes, neutrophils and macrophages to facilitate the removal of immune complexes. Pre-T cells originate in the bone marrow and travel to the thymus where they develop into one of the three classes of a mature T cell: cytotoxic (Tc), regulatory (Tr) or

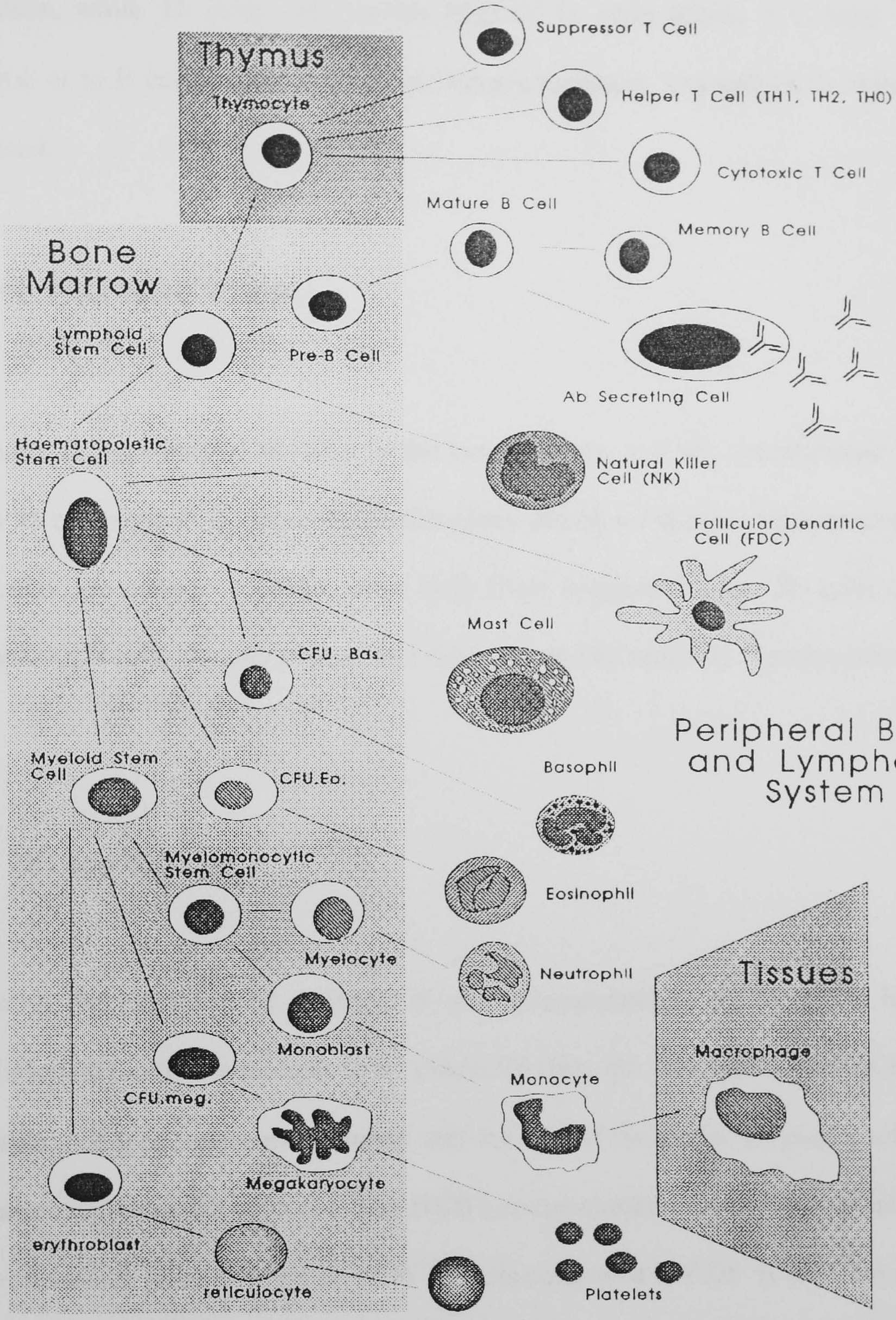


Figure 1.1: Cells of the immune system (Immunology. 3rd edition. Kuby. 1997).

helper (Th) T cell. Tc cells, as mentioned above, take part in the cellular immune responses, while Th cells can provide help to Tc cells during a cellular immune response or to B cells during a humoral immune response. The role of Tr cells is later discussed.

1.3 B LYMPHOCYTES.

In adults, B lymphocytes develop in the bone marrow and are characterised by their ability to rearrange their immunoglobulin genes. B-cell surface bound immunoglobulin molecules are antigen specific. With help from antigen-specific Th cells (cognate interaction), B cells clonally expand and differentiate into antibody forming cells (AFC).

1.3.1 B1 and B2 Cells.

Recently, the existence of different B-cell subpopulations (B1 and B2) has been established. B1 cells are thought to be generated from the same precursors as B2 cells. Precursor cell type, microenvironment and the age of the donor all play a role in the generation of B1 or B2 cells (1). The B1/B2 nomenclature replaces an earlier scheme based on surface phenotype alone: $CD5^+$ B cells compared to $CD5^-$ B cells. B1 cells are mainly present in the peritoneal cavity of mice and humans and are characterised by the highly autoreactive immunoglobulins on their surface which are thought to play a role in the positive selection of this cell subpopulation. B2 cells die rather than become self-

reactive (2). B1 cells can be further distinguished from B2 cells as: IgM^{hi} IgD^{lo} CD23- CD43+ and CD5+ (B1a) or CD5- (B1b) cells (3).

B1 cells contribute to the production of serum immunoglobulin and natural autoantibody. The main antibody isotypes secreted by B1 cells in serum are IgG3 and IgM, although it has been shown that B2 cells can contribute to the secretion of other serum immunoglobulins, probably as non-specifically promoted by T cells or cytokines (4). Both B1a and B1b B cells reconstitute and generate IgA plasma cells in the lamina propria (5). Finally, the involvement of B1 cells in limiting tumour development has been suggested. In peritoneal B1a cells, consitutive expression of STAT3 oncogene seems to play a role in the B1 cell oncogenesis function. Expression of this gene is probably due to the constitutive cross-linking of the BCR by self-antigen and the unique cytokine exposure in the peritoneal cavity (6). Recent results emphasise the importance of B1 cells (and marginal zone B cells), in T-cell-independent antibody responses, thereby contributing to innate immunity (7), (8).

1.4 The Immunoglobulin Molecule, Mediator of Humoral Immunity.

The basic immunoglobulin unit is a tetrameric glycoprotein composed of two identical light and two identical heavy polypeptide chains which are approximatelly 220aa (25 kDa) and 440aa (50 kDa) each respectively. Molecular weight depends on the Ig heavy chain class, carbohydrate content and whether the Ig molecule is the membrane bound or the monomeric/multimeric secreted form (table 1.1).

The immunoglobulin light chain consists of two domains: a constant (CL) and a variable (VL) domain. The heavy chain consists of a variable domain (VH) and three or four constant domains (CH1, CH2, CH3, CH4) depending on the Ig class and subclass. In some Ig molecules there is also a region called the hinge which is interposed between the CH1 and CH2 domains. In addition, depending on whether the molecule is membrane bound or secreted, a transmembrane region or a joining region may be situated next to the CH3 or CH4 domain. One light chain is bound by a disulphide bond (S-S) in the CL domain to the CH1 domain of a heavy chain, and two heavy chain/light chain dimers are bound together at the hinge by between 1 and 11 disulphide bridges to create the tetrameric unit described before. IgM is an exception as it does not have a hinge and uses rigid CH2 domains in close association as a hinge-like structure, and its heavy and light chain dimers are disulphide bonded between the CH2 and CH3 domains.

Each heavy or light C and V domain contains an internal disulphide bond which stabilises the characteristic immunoglobulin fold tertiary-like structure. The gene sequence coding for the immunoglobulin fold structure is conserved and other cell surface molecules using it are said to be members of the immunoglobulin gene superfamily. In addition to covalent bonds, the Ig heavy and light chains are held together by non-covalent interactions between the Ig gene domains. N-linked carbohydrate chains are also linked to the immunoglobulin domains at various positions according to the Ig class, and these contribute to the structure and function of the molecule. Human IgG subclasses have one N-linked carbohydrate chain bound to each CH2 domain. This interferes with the non-covalent interactions of the CH2 domains and

Table 1.1: PHYSICO-CHEMICAL AND FUNCTIONAL PROPERTIES OF THE HUMAN IMMUNOGLOBULIN CLASSES

Property	IgM	IgD	IgA	IgE	IgG
Ig Heavy chain class	μ	δ	α1 or α2	ε	γ1, γ2, γ3 or γ4
Molecular weight (kDa)	970[a]	184	385[b]	188	150
Molecular weight of heavy chain (kDa)	65	69.7	56 or 52	72.5	53
Number of heavy chain domains	5	4	4	5	4
Ig carbohydrate content (%)	12	9-14	7-11	12	2-3
Ability to fix complement	+	-	-	-	+
Ability to cross placenta	-	-	-	-	+
Staphylococcal protein A binding	-	-	-	-	+
Binding to mononuclear cells	-	-	+	+	+
Binding to neutrophils	-	-	+	-	+
Binding to mast cells and basophils	-	-	-	+	-*
Binding to T and B cells	+	-	+	+	+
Binding to platelets	-	-	-	?	+

a pentameric form, **b** dimeric form

* Only IgG4.

the molecule bends outwards. This change in the 3-D structure may influence the effector function of the heavy chain.

Enzyme digestion splits the immunoglobulin molecule into functional components. There are two identical Fab fragments (antigen-binding), which donate to the molecule a bi-valent antigen binding capacity. The V domains of heavy and light chains contain hypervariable regions: CDR1, CDR2 and CDR3. When the V domain immunoglobulin fold is arranged, these regions form extra polypeptide loops which associate non-covalently to form the antigen-binding pocket. The CH1 domain of the Fab also contains a binding-site for complement C4b/C3b. The Fc region (fragment crystalline) contains binding sites for complement C1q (CH2), Fc receptors on macrophages and monocytes (CH2), Fc receptors on neutrophils and NK cells (CH2 and CH3) as well as staphylococcus protein A.

1.4.1 Immunoglobulin Heavy Chain Classes.

Different heavy chains confer different effector functions. In human and mouse there are five major classes of immunoglobulin heavy chain constant regions: M, D, G, A and E. The G class is further divided into: G1, G2, G3 and G4 in the human and G1, G2a, G2b and G3 in the mouse. Humans also has two subclasses of A: A1 and A2.

1.4.2 IgD.

In mature B cells, the CHδ genes are transcribed with the CHμ genes in one long mRNA transcript (9), (10). Alternative splicing allows IgD to be co-expressed with IgM in the membrane, but never alone. IgD is present in human serum at a low concentration (approx. 30μg/ml), but as IgD is not secreted, this may be due to proteolytic cleavage or loss from the cell surface of activated B cells. The function of IgD is controversial. It is thought to play a role in immune tolerance, B-cell activation and memory (11). However, in the IgD^- mouse, it is not essential for B-cell maturation (12). Instead, it may act as a more efficient antigen capture molecule than IgM because of its greater hinge flexibility (13). IgD is not secreted because the δ heavy chain gene lacks a secretory protein coding region. In spite of this, some myelomas (1%) secrete IgD. This may be explained by unequal crossing over of sister chromatids and insertion of the Cμ secretory exon to the 3' of Cδ (14).

1.4.3 IgM.

IgM is the first Ig isotype expressed on the surface of B cells. It is the first antibody produced during an immune response before the switch to IgG, IgA or IgE and is present in human serum at approximatelly: 1.5-2.0mg/ml. Secreted IgM is a pentameric molecule. IgM molecules are joined by disulphide bonds between CH3 and CH4 domains, and an additional protein called the joining (J) domain. Although individual IgM molecules are often of low affinity, their pentameric structure compensates for this

by having a high antigen avidity. IgM is also the only Ig molecule apart from IgG which can fix complement.

1.4.4 IgA.

IgA is present in normal human serum at approximatelly: 1.4-4 mg/ml. The IgA molecule is present as a monomer in serum, but can be secreted in body fluids (e.g.: milk, intestinal secretions, saliva, tears) as dimers where it binds to invading pathogens as a first line of defence. Dimeric IgA is linked by one CH3 domain to a joining (J) protein. An additional protein polypeptide called the secretory component is wound around the dimer and disulphide bonded to each end to one CH2 domain of each IgA molecule conferring resistance to GI tract enzymes. IgA-antigen complexes can bind to FcαRs on human monocytes, granulocytes, neutrophils and subpopulations of T and B cells (15), and mediate phagocytosis and superoxide production for bacterial cell lysis and killing. The human IgA subclasses: IgA1 and IgA2 are found at a ratio of 9:1 in normal human serum. This concentration difference could be due to the spatial distance between the Cα2 gene and the VDJ genes. Cα2 bearing the most 3' of the Ig heavy chain constant region genes. In external secretions however, IgA2 can rise up to 50% of serum IgA levels. This may be accounted for by the susceptibility of IgA1 to proteolysis. IgA2, which has lost a large part of the IgA hinge region (13aa), is resistant to a number of bacterial proteases which attack IgA1 in external secretions, especially in the gut (16). Interestingly, the rabbit has evolved 13 IgA subclasses which differ from each other in

their hinge and CH1 regions, perhaps endowing them with protection against a wide range of bacterial proteases. The rabbit is a herbivore with a large bacterial harbouring caecum and appendix and would gain considerable selective advantage from developing protective IgA (17).

1.4.5 IgG Subclasses.

The emergence of the human IgG subclasses has given a selective advantage to defence against invading pathogens (figure 1.2). IgG subclass heavy chain constant region genes are encoded by distinct gene loci. Their functional differences are a direct result of structural changes in the hinge regions brought about by changes in the amino acid sequence (18). The hinge is extremely important in the effector functions of the Ig molecules. Segment flexibility correlates with the ability to bind complement. If the hinge is deleted, the IgG molecule becomes a rigid T-shape which is unable to activate complement or bind to monocytes (19). IgG1 has an extended hinge region with non-coplanar Fab arms. IgG3 has an extremely long hinge region which has arisen by multiple duplications of the original hinge exon. Both IgG1 and IgG3 have flexible hinge regions and are good mediators of effector function. Both IgG2 and IgG4 have short hinge regions and are poor mediators of effector function. The IgG2 hinge is particularly rigid and inflexible due to the lack of glycine residues. The classical pathway of complement is activated by binding of the C1q to the CH2 of the IgG molecule. The flexibility of the IgG1 and IgG3 Fab arms allows optimal binding for C1q while the rigidity of IgG2 increases steric hindrance. IgG4 does not bind C1q and does

not activate complement. This is probably because the IgG4 Fab arms are too close to the Fab binding site on the Fc region.

IgG subclasses bind to Fcγ receptors with variable affinity (IgG1>IgG3>IgG4>IgG2). This is not due to the structure of the hinge or flexibility of the Fab arms, as all Fcs bind with the same affinity. There are three Fcγ receptors: FcγRI (CD64), FcγRII (CDw32) and FcγRIII (CD16) (20). CD64 is the high affinity Fcγ receptor. It is found on macrophages, monocytes, neutrophils and is able to bind both monomeric and polymeric IgG. The CH2 domain of IgG1 binds to CD64 and mediates antibody dependent cytotoxicity and clearence of immune complexes. CD32 has a low affinity for aggregated IgG and exists as A, B and C forms. All three are found on monocytes and endothelial cells, the B form occurs on B cells and A and C forms on neutrophils. IgG binding to this receptor mediates phagocytosis and the oxidative burst in monocytes and phagocytes, transport of IgG across the epithelial placenta and delivers a negative signal to B cells in combination with anti-Ig. CD16 also has low affinity for aggregated IgG and occurs in two forms. It is a transmembrane molecule on NK cells and macrophages, with its glycosylphosphatidylinositol (GPI) linked on neutrophils. GPI-linked CD16 is thought to work in concert with CD32 as a capture molecule for aggregated IgG (21). IgG-antigen complexes binding to the transmembrane mediates phagocytosis and cellular cytotoxicity.

In vivo, IgG subclasses are directed against particular pathogens, to confer optimum protection. In the human, IgG1 and IgG3 subclasses are raised against extracellular and intracellular viral antigens (22). IgG2 is the major IgG-subclass raised against

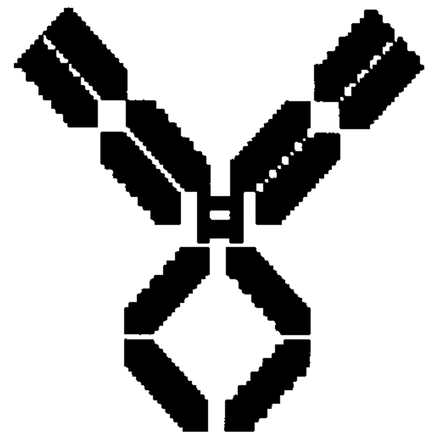
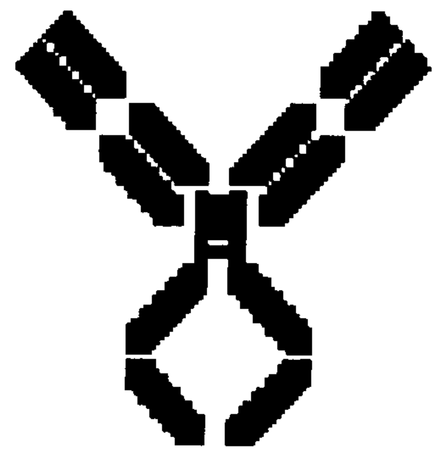
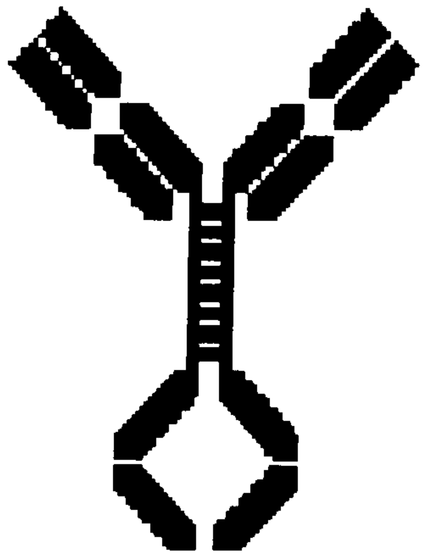
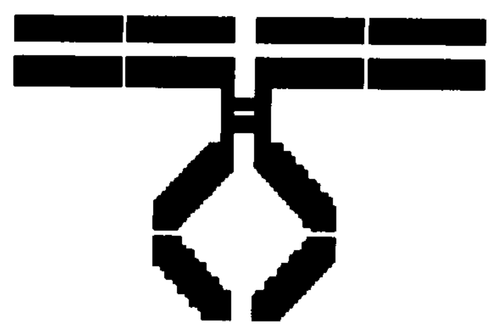
	Hinge	Complement activation	Function	Structure
IgG1	**Flexible**	+++	General immunity	
IgG2	**Inflexible**	±	Neutralisation - bacteria	
IgG3	**Flexible**	++++	Viral immunity	
IgG4	**Rigid**	-	Neutralisation – soluble antigen	

Figure 1.2 : Human IgG subclasses 1-4.

carbohydrate antigens, especially bacterial polysaccharides. Protection by IgG2 is seen in adults, but in young children IgG1 is protective against both protein and carbohydrate until the IgG2 response matures and takes over. IgG1 is less efficient than IgG2 at combating encapsulated bacteria (23). Protection against acute and early parasitic infection is directed mainly by IgG1, (normal responses to larval antigens), followed by IgE and IgG4 responses after long term exposure (24). IgG4 also increases in cases of chronic antigenic stimulation (allergy) (25), (26) and (27)), and confers long term immunity to antigen (28) and (29).

1.4.6 IgE.

IgE is present in normal human serum at very low concentrations (5-150 ng/ml) (30). Binding to the high affinity IgE receptor (FcεRI) on basophils and mast cells causes degranulation and release of histamine, production of prostaglandins and leukotriene. Binding to the low affinity IgE receptor (FcεRII, CD23) on B cells, T cells and a number of other immune cells increases antigen processing (31) and induces IgE-dependent cellular cytotoxicity, lysis and clearance (32). IgE is also an activator of allergic responses. Loss of control of IgE production leads to excessive serum IgE and binding to FcεRI on mast cells and FcεRII on B cells. Cross-linking with allergen on FcεRI leads to immediate type hypersensitivity reactions, release of mediators and allergic responses (33), (34). Binding of IgE-antigen complexes to FcεRII increases antigen processing by plasma cells and prolongs the allergic immune response (35).

1.4.7 Immunoglobulin Light Chains

There are two light chain loci in the human and mouse genomes. κ and λ. These have multiple V (variable) and J (joining) genes which encode the VL domain and C regions for the CL domain (the κ locus has only one C gene). V and J genes are rearranged at random so that in a given number of B cells, many different combinations are produced. The hypervariable regions: CDR1, CDR2 and CDR3 are encoded within the V genes, and undergo somatic hyper-mutation after antigenic stimulation of the B cell. This is important in the process of Ig affinity maturation in the germinal centre where antigen binding specificity is increased (36), (37) and (38).

1.4.8 Immunoglobulin Heavy Chains

There is only one locus for the immunoglobulin heavy chain in humans and mice. The locus consists of multiple V, D (diversity) and J genes which make up the VH domain, and C genes which encode the CH domains. V, D and J genes are rearranged at random in a similar way to the light chains, except that the presence of D regions creates an even greater diversity for Ag recognition. In the heavy chain, CDR1 and CDR2 regions are encoded by the V genes while the CDR3 region is encoded by the VDJ genes.

1.4.9 The Heavy Chain Constant Region Genes.

There are eight functional heavy chain constant region genes in the mouse encoded by: Cμ, Cδ, Cγ3, Cγ1, Cγ2b, Cγ2a, Cε and Cα. Humans have nine functional and two non-functional pseudogenes encoded by: Cμ, Cδ, Cγ3, Cγ1, ψε, Cα1, ψγ, Cγ2, Cγ4, Cε and Cα2. Heavy chain constant region genes are not rearranged in immature B cells, but different heavy chain genes are expressed in B-cell maturation by a process called Ig class switching.

1.5 B Cell Ontogeny and Antigen Independent Maturation.

B-cell progenitor maturation is signalled by an antigen-independent ordered cascade of immunoglobulin gene rearrangements accompanied by the differentially controlled expression of cell surface antigens (39) (Figure 1.3 and 1.4). Pro-B cells are the first identifiable B-cell precursors. They express MHC class II, CD10 and CD34 but their heavy chain genes still retain germ line configuration on both chromosomes.

Heavy chain gene rearrangements take place first on both maternal and paternal chromosomes. The sequence of gene rearrangement has been clarified by the use of murine B-cell lines, plasmacytomas, hybridomas and transformation of mouse pre- B cells by AMuLV (40), (41) and (42). First, D to J gene joining takes place. This is followed by V to DJ gene joining to give a VDJ rearranged gene which can be

transcribed with the heavy chain constant region gene, Cμ, to give a productive VDJCμ mRNA (43). Productive mRNA, with VDJ transcribed in a viable reading frame, is able to produce a protein product which can be expressed, first as μ heavy chain in the cytoplasm, and later on the cell surface with the pseudo-light chain or κ and λ (44).

Pseudo-light chain genes, which do not rearrange, are expressed early in the Pre-B cell (45). Their protein products may be associated with the newly synthesised μ heavy chain to form a cytoplasmic or membrane-bound complex. This occurs before the appearance of immunoglobulin light chain rearrangements (46), (47) and (48). The heavy chain V region of the pre-B cells (VpreB) has sequence homology to the light chain V regions, while λ5 and λ14.1 are homologous to the light chain J and C regions. The protein product of λ5, ω, is disulphide bonded to the μ-heavy chain and the protein product of VpreB, ι, is held in a non-covalent association to form a heavy/pseudo light chain heterocomplex (49).

Appearance of the heavy chain protein product on the B cell surface, either alone, or possibly in association with the pseudo light chain components, prevents further heavy chain gene rearrangements on the other allelic chromosome (Figure 1.3a). The B cell may be held in stasis at this point in maturation as a self-renewing pool. When further maturation is required, a signal, possibly through a surface pseudo/light chain-heavy chain complex, may inhibit further transcription of ι and ω, and induce light chain rearrangements (50) (Figure 1.3b). κ light chain genes rearrange first in a similar fashion to the heavy chain genes. If the κ light chain product is non-functional, then λ genes rearrange. Isotypic exclusion is responsible for which of the four Ig light chain alleles is expressed on each B cell (2κ and 2λ). Light chain products become associated with

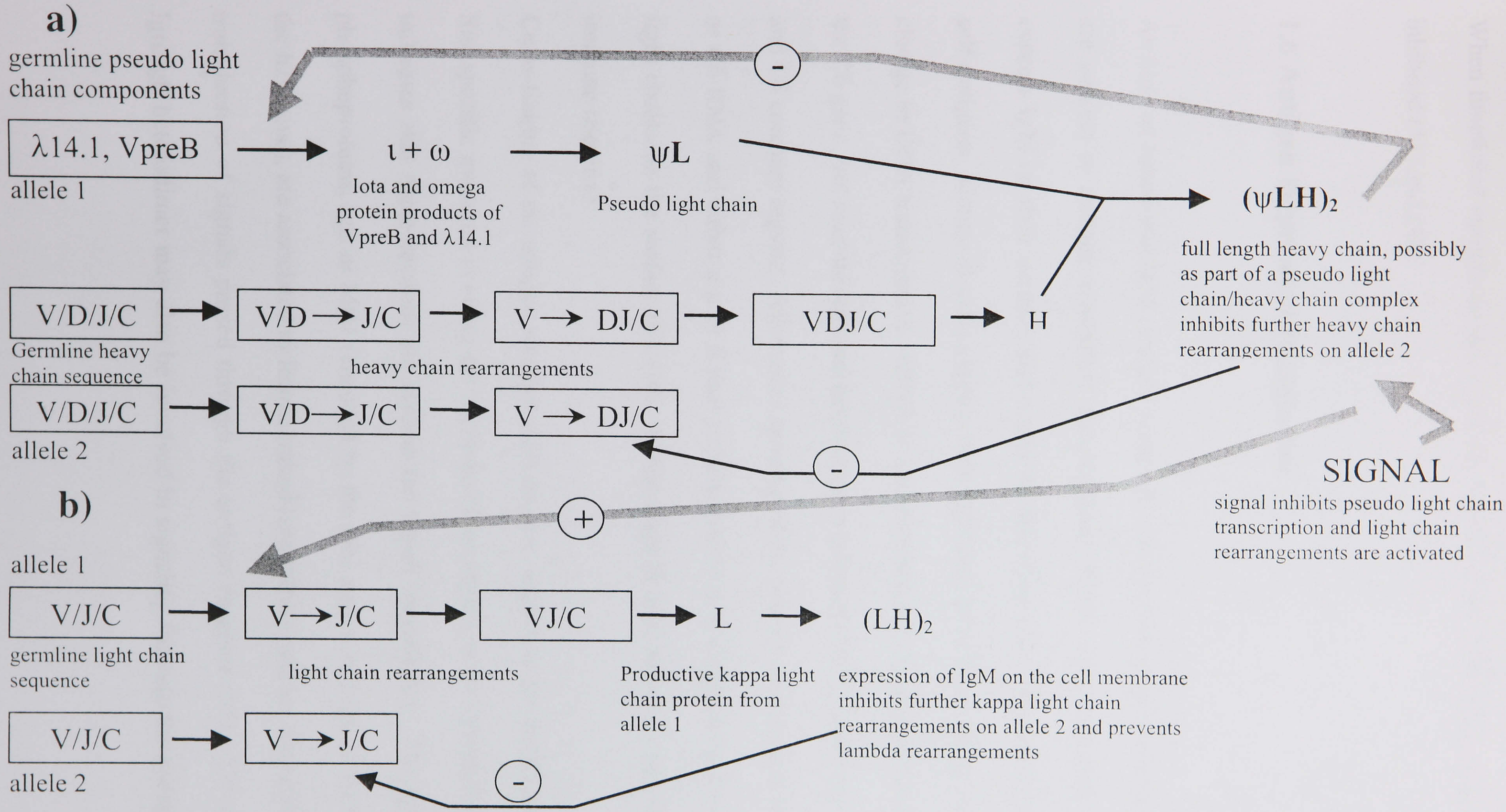


Figure 1.3: Events leading to IgM expression on the immature B cell. a) Heavy chain rearrangements and expression of pseudo light chain components. b) Light chain rearrangements and expression of the mature IgM molecule.

heavy chains in the endoplasmic reticulum (ER) and are transported to the cell-surface. When functional antigen receptors are expressed, further light chain rearrangements are inhibited (51) and (48).

1.6 Antigen Dependent Maturation

As soon as functional IgM (antigen receptor) is expressed at the B-cell surface. B cells are subject to antigen controlled development (figure 1.4). Immature B cells only express IgM on their surface, and it may be this stage that is important in tolerance to self-antigens. Mature B cells express both IgM and IgD. This change is marked by a change in DNA transcription. mRNA is not terminated in the μ stop codon to the 5` of the Cδ gene, but read-through occurs creating a primary mRNA transcript with both Cμ and Cδ constant regions. Alternative splicing of the primary transcript deletes either Cμ or Cδ RNA and either a μ or δ heavy chain protein is synthesisied and expressed with light chains on the surface (9), (10). At this stage B cells may take part in the primary immune response.

Cross-linking of the antigen receptor with antigen, signals to the B cell and activates it. Site specific mutation in either the transmembrane region or the cytoplasmic tail of IgM indicates that these sections are critical for signal transduction. The IgM associated phosphoproteins, Igα or MB1 (encoded by the mb1 gene), and Igβ or B29 (encoded by the b29 gene), are associated to form a linked heterodimer and are responsible for signal transduction of signals passed through the antigen receptor (52), (53) and (54). The Igα/Igβ heterodimer may also be involved in signaling through the pseudo/light chain

complex in pre-B cells (55). The MB1 protein may be required for transport and insertion of these complexes into the membrane (56). Although the IgM and IgD complexes on the mature B cell share the same associated proteins. other proteins may be present which confer different biological responses for tolerance. activation and memory mechanisms (57).

Cross-linking of the Ig molecule with antigen and activation by T cells through cell/cell interaction and also by cytokines, causes B-cell activation, proliferation and loss of IgD (57) and (58). Under the correct conditions. B cells will differentiate into antibody secreting cells (AFCs) or plasma cells. The shift from membrane IgM (mIgM) to secretory IgM (sIgM) is achieved at the RNA processing level and by post-translational regulatory control (59).

Activated, mature B cells primarily secrete IgM. the first immunoglobulin to be secreted in an immune response. IgM+ B cells can also undergo heavy chain class switching to become IgA, IgG and IgE AFCs and to generate IgA+. IgG+ and IgE+ memory B cells. The function of immunoglobulin heavy chain class switching is to transfer an antigen binding site to a class of heavy chain which gives maximum protection against a specific pathogen. Immunoglobulin class switching is mainly controlled by Th cells via cell/cell interactions as well as by the various T-cell derived cytokines.

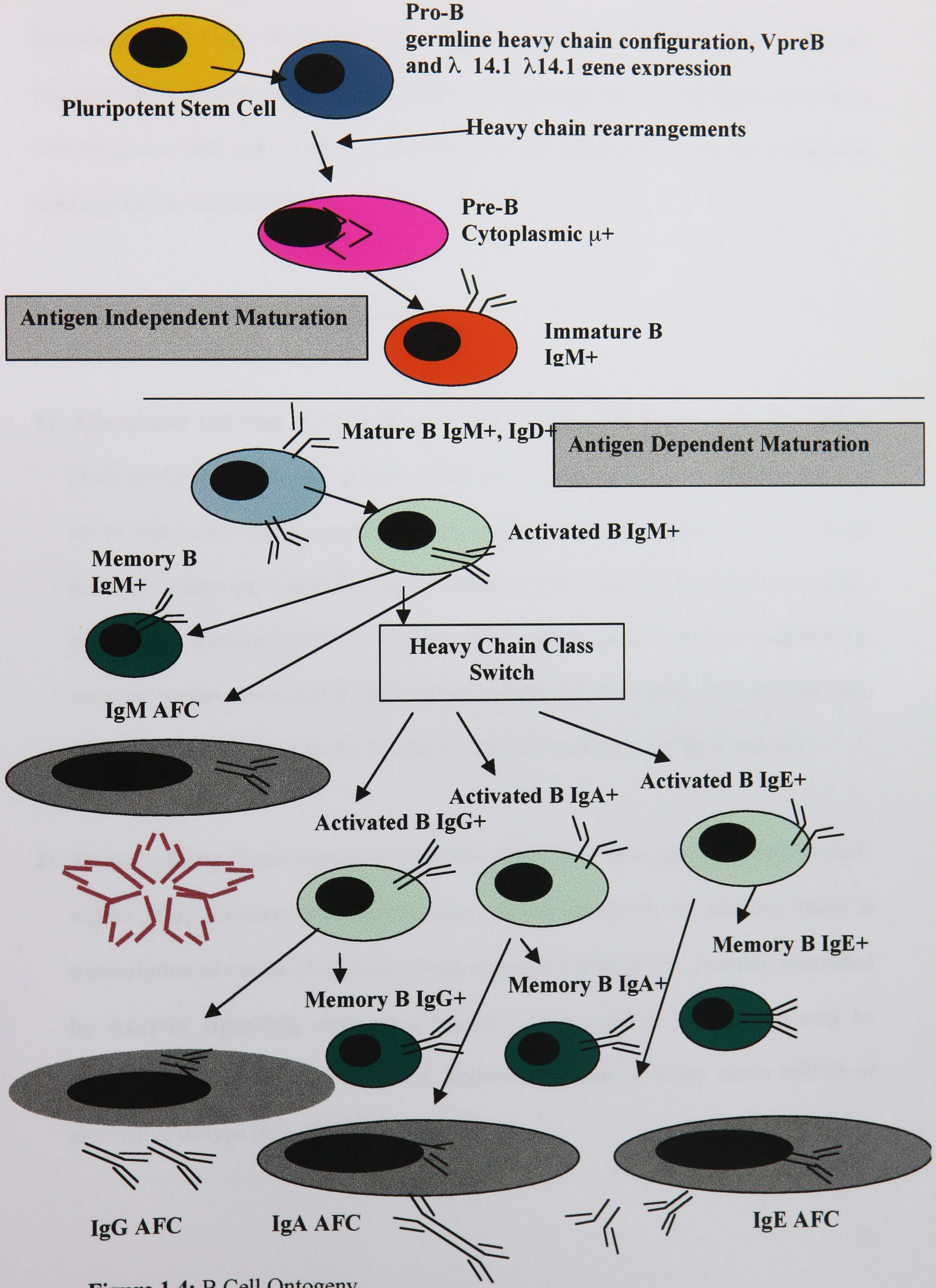


Figure 1.4: B Cell Ontogeny

1.7 Mechanisms of Immunoglobulin Heavy Chain Class Switching

Immunoglobulin heavy chain class switching allows an Ig molecule to change effector function whilst retaining specific high affinity antigen binding sites. Ig heavy chain class switching can take place either at the RNA or the DNA level. The most common mechanisms are outlined below.

1.7.1 Class Switching at the RNA level.

1) **Alternative Splicing.** Transcription of part or the entire heavy chain locus takes place producing a very long primary RNA transcript containing the VDJ and some or all of the heavy chain constant region genes. Alternative splicing of the large primary transcript, controlled by receptor/ligand binding responsive elements, produces a truncated mRNA with the VDJ region spliced onto the appropriate constant region exons, and deletion of the intervening RNA (60), (61), (9) and (10). This mechanism is used by the B cells for the dual expression of IgM and IgD.

2) **Trans-Splicing.** Transcription of the heavy chain VDJ locus and one heavy chain C region (Cμ) produces a complete heavy chain pre-RNA. In addition there is transcription of one or more heavy chain constant region genes, possibly controlled by receptor signalling response elements. The pre-RNA VDJ region may be transpliced onto another pre-RNA C region to produce a heavy chain mRNA of alternative isotype (62), (63), (64) and (65).

1.7.2 Class Switching at the DNA Level.

1) Unequal Sister Chromatid Exchange and Homologous Chromosome Recombination.

Both these mechanisms involve unequal crossing over and recombination of DNA at sites of homology upstream of each heavy chain constant region gene. Sister chromatid exchange involves recombination of sister or duplicated chromosomes during metaphase. Homologous chromosome, or trans-switch, recombination involves recombination between the active and inactive allelic chromosomes either prior, or during mitosis. Both mechanisms result in the removal or transfer of C genes to the productive allele, changing the class of heavy chain transcribed (61) and (66).

2) **Site Specific Recombination: The Accessibility Model of Ig Heavy Chain Class Switching (Figure 1.5).** Signalling through a cell surface receptor activates a responsive element which initiates the opening of, or accessibility to, the DNA at specific S (switch) sites. S sites are tandemly repeating core oligonucleotide sequences stretching from 1kb (Sε) to 10kb (Sγ) which are located 5' to each heavy chain constant region gene except for IgD (67), (68) and (69). Initial opening of the DNA at the S sites allows transcription of the individual constant region genes, producing non-productive, germline RNA (sterile transcripts). Switch site recombination may occur via a common switch recombinase enzyme, whose access to the DNA is carefully controlled, instead of site specific switch recombinases which are controlled individually. All heavy chain class switching may be affected by a common recombinase but under specific control by cytokine responsive

elements (61). Active cell division or mitosis, involving the unwinding of chromatin during chromosome replication, may be needed to allow coming together, homologous recombination of the S sites and deletion of the intervening circular DNA (70), (71) and (67). Cytokine responsive elements could control heavy chain class switching by increasing DNA accessibility upstream of specific heavy chain C region genes. In humans, a novel IL-4 responsive element has been found 5' to the ε switch site which appears to play an important role in sterile transcript induction and switch recombination (72). Accessibility can be induced by creating: a) nucleosome free DNase I hypersensitive sites for recombinase or RNA polymerase access (73) or b) demethylation of the DNA at the switch sites. The Ig and T-cell receptor (TcR) genes are methylated in germline and lymphoid cells before activation and become demethylated after demethylation of the lymphoid recombinogenic sequences (e.g. Ig switch regions). This phenomenon is highly specific to cells of the lymphoid lineage and such sequences are normally demethylated in other lineage cells such as hepatocytes and kidney cells. There is significant correlation between demethylation and Ig-gene transcription in the B lymphocytes (74). Finally, it is thought that sterile transcripts themselves may play more than a by-stander role in the Ig-heavy chain class switch mechanisms. Sterile transcripts occur in both mouse and human B cells (75), (76), (77) and (78), and may be important regulators of heavy chain class switching (79). Sterile transcripts may be trans-spliced into the VDJ of the current heavy chain RNA to allow transcription of the new Ig isotype alongside the old, before DNA recombination takes place (80). This would create intermediary double isotype cells (63). Sterile transcripts may also a) interact with targets important in targeting the locus for recombination and/or b) interact with the DNA and stabilise

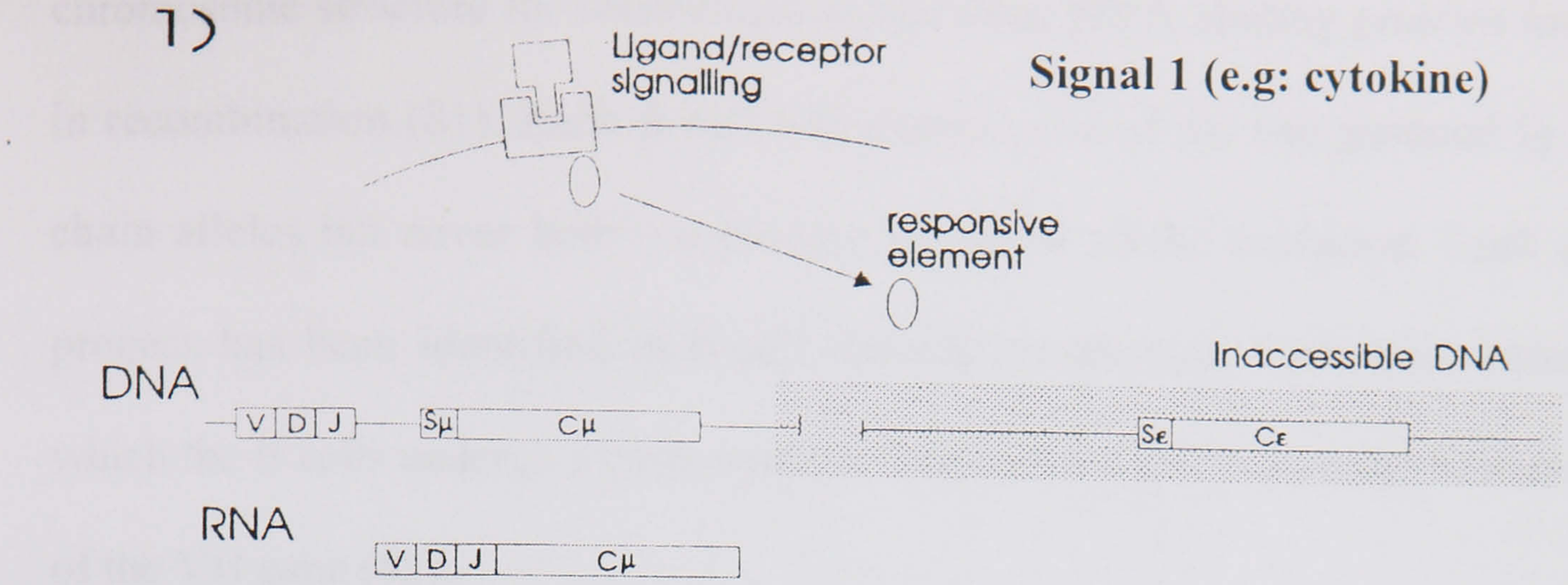


Figure 1.5 : Accessibility model of heavy chain human Ig class switching (reviewed by Diaz-Cano. 1996).

chromosome structure for recombination and other DNA binding proteins involved in recombination (81). Each B cell will express one of the two parental Ig heavy chain alleles but never both – a process known as allelic exclusion. Lack of this process has been identified in B-cell chronic lymphocytic leukaemia patients in which the B cells undergo a class switch of one allele with the incomplete expression of the VH gene (82).

1.8 IgE and Allergy.

Over the past three decades, allergy has increased in prevalence within both industrialised and developing nations (83) and (84). Approximately 30% of the United Kingdom population suffers from some form of allergic disease, a statistic attributed to a plethora of reasons including increased diesel pollution (85) and (86), increased use of carpets which provide a comfortable home for house dust mites (87), and a decrease in early childhood infections which could be vital in preparing the immune system for later life and the inevitable encounter with potential allergens (88).

Study of the involvement of the immune system in allergic disease was revolutionised in the 1960's by Ishizaka and colleagues with the discovery of immunoglobulin E (89) and (90). However, clinical investigation into the mechanisms of allergy had begun fifty years earlier in 1921, when Prausnitz discovered factors in human serum which could transfer skin sensitivity from an allergic donor to a non-allergic recipient, later termed

the Prausnitz-Kustner test (Prausnitz 1921). Confirmation of the pivotal role of IgE in the pathology of allergic diseases was supported by clinical investigations into hayfever (91), (92) and dust mite allergy (93).

1.8.1 Mechanism of IgE function.

The function of IgE is ultimately dependent on a cell's IgE receptor expression profile. Two types of IgE receptor have been identified. Mast cells and basophils express the high affinity receptor (FcεRI) on their surface (94), the binding of which by IgE primes these cells to secrete, upon subsequent exposure to specific antigen, a panel of proinflammatory mediators. These can be immediate (histamine and heparin) or can be secreted at a later stage (leukotrienes, prostaglandins and cytokines). Cytokines such as IL-4, induces B-cell isotype class switching, IL-3 favours the maturation of mast cells and basophils, and IL-5 stimulates eosinophil infiltration (95). B cells, T cells, monocytes, eosinophils and some epithelial cells can express the low affinity IgE receptor FcεRII, also known as CD23 (96). In humans there are two forms of CD23 which differ in their intracellular amino terminal 6/7 amino acids. The A form corresponds to murine CD23, whereas the B form is also found on other haematopoietic cells including T cells. CD23 has been shown to have several different functions which are cell type specific. On monocytes, platelets and eosinophils CD23 mediates IgE-dependent cytotoxicity against parasites. CD23 expression on B lymphocytes is involved

in the IgE specific internalisation of antigens for antigen processing and presentation to T cells. CD23 has also been shown to mediate the release of inflammatory mediators from macrophages and to be involved in signal transduction. The enzymatic cleavage of FcεRII has been shown to produce a soluble form of the receptor which can regulate human IgE production and promotes T and B lymphocyte proliferation (97). This indicates the role of sCD23 as an autocrine B-cell factor. Recent work suggests that some allergens. such as phospholipase A_2 from bee venom (98) and Der p 1 from dust mite (99), (100) and (101). in addition to acting as immunogens, have the intrinsic ability to subvert the regulatory process of IgE synthesis, therby favouring an allergic outcome. Indeed, Der p 1 has been found to have a digestive function (102), cleaving CD23 from the surface of cultured B cells at two sites (103). This has been also confirmed "in vivo" by the use of mouse models. The upregulation of IgE via the digestive ability of Der p 1 can be also indirect as the latter cleaves CD25, the α-subunit of the IL-2 receptor, leading to a predominate Th2-type response with high levels of detectable IL-4 and IL-5 (104).

1.8.2 Generation of IgE.

For B cells to synthesise IgE, genetic rearrangement of immunoglobulin genes is required. The end product of these gene rearrangements is transcribed into mRNA transcripts encoding the constant and variable regions of the immunoglobulin molecule.

The variable regions of immunoglobulins are encoded by multiple germ line transcripts that are assembled into complete V(D)J regions by recombinase enzymes (105).

The isotype switching process facilitates the linkage between an antigen specific variable region of the immunoglobulin and different heavy chains which determine the functionality of the final immunoglobulin produced. The recombination mechanisms which mediate this process delete regions between the V(D)J segments and the genes encoding the various heavy chain segments (CH genes). The recombination events are initiated at highly repetitive regions of the genome known as switch (S) regions which lie 5' to the respective heavy chain region. At the level of the gene, isotype switching is thought to be regulated by factors which cause local alterations in chromatin structure thereby increasing the accessibility of the various S regions to switch recombinases (106).

Isotype switching is preceded by the production of a germ line transcript immediately upstream of the CH gene. These transcripts contain several stop codons and are consequently never translated into proteins, hence they are known as "sterile transcripts". Whilst their role in switch recombination remains unclear, their production is essential for the process to occur (107), (108).

At a cellular level, B cell immunoglobulin class switching is regulated by cytokines of which two have been shown to selectively promote switching to IgE, IL-4 (109) and (110) and IL-13 (111). Treatment of B cells with IL-4 has been shown to initiate the synthesis of a 1.7-1.9 kb sterile transcript of the Cε region containing a 2kb region

upstream of the ε switch region, and the four exons encoding the heavy chain domains of IgE (75). Ligation of the IL-4 receptor α-chain leads to STAT6 and IRS-2 activation, which, in combination with CD40 / CD40L interaction leads to IgE class switching (112). IL-13 expression by allergen stimulated T cells is also increased in allergic disease and is associated with higher levels of eosinophil stimulating cytokine IL-5 (113). Other cytokines such as IL-10 and TGF-β are reported to promote switching to IgG1 and IgG3 (114), (115) and IgA (116) respectively. IFN-γ, IL-10 and IL-2 have been shown to inhibit IgE transcription (117), (118), and IL-5 and IL-6 can enhance IL-4 induced IgE production (119). IL-4 was shown to be essential for IgE synthesis *in vivo* in mice infected with the nematode *Nippostrongylus braziliensis* where anti-IL-4 (120), and anti-IL-4 receptor (IL-4R) antibody, inhibited IgE synthesis, though these studies also raised the possibility of STAT6 independent pathway of type 2 response (121). Ongoing IgE responses in animals sensitised with the nematode *Heligmosomoides polygyrus,* which induces a chronic IgE response, were inhibited with anti IL-4 (120) indicating that IL-4 is required for continued IgE production. The requirement of IL-4 for IgE synthesis was confirmed in IL-4 knockout mice (122) which were unable to produce IgE. It is clear from these observations that type 2 responses favour IgE production whilst type 1 responses promote the production of other immunoglobulin isotypes.

1.9 IgE Switching

Induction of IgE in human cells involves three distinct steps: a) induction of ε germ-line transcription, b) DNA recombination and c) mature RNA transcription/translation (123). The study of switching to a particular isotype such as IgE, is rather difficult as freshly isolated polyclonal B cells are required that should be homogeneous in their genetic make-up. Ideally, such monoclonal cells should be sIgM+, sIgD+, they should undergo a high rate of switching to all downstream Ig classes in response to physiological stimuli, possibly in a cytokine-directed fashion, and finally they should display the phenotypic changes that are putatively attributed to B cells that switch and progress through the germinal centres. No B-cell line with all these properties has been reported yet.

The importance of IL-4 and anti-CD40 in IgE B cell switching is well established (124). IL-4 plays multiple roles in the induction of IgE synthesis. It promotes IgE switching, by enhancing the accessibility of the ε-switch region to the common recombinase (125). Furthermore, it increases the expression of MHC class II antigens on B cells, enhancing their antigen-presenting capacity (126) and (127). Thus, IL-4 would promote T/B cognate recognition and at the same time drive the B-cell response towards the IgE isotype. The possibility exists that the CD40 mAb promotes IgE synthesis in the presence of IL-4 because it is so effective in keeping the B cells growing in culture for a long time, thereby giving the B cells time to switch or to divide and produce IgE.

The signals required for isotype switching are provided to the B cell by a complex series of interactions with an antigen specific T cell (124). Engagement of the T-cell receptor/CD3 complex by MHC class II molecules results in the rapid expression of

CD40L, which engages CD40, the counter-receptor constitutively expressed on the surface of B cells (128). T/B cell interactions mediated by CD40/CD40L are amplified by interactions of accessory molecules, particularly the CD28/B7 ligand/receptor pair. The sequence of events taking place during the interaction between T and B cells, leading to IgE switching, are shown in figure 1.6. Interaction between CD40 and CD40L generates the secondary signal (signal 2) for the switching process (129). Engagement of CD40 results in the expression of B7 molecules on B cells. B7 engages CD28, inducing high rate of secretion of IL-4 by the T cells (130), (131) and (132). IL-4 binds to the heterodimeric IL-4 receptor (IL-4R), which consists of an α chain (shared by the IL-13R) as well as a γ chain (shared with the receptors for IL-2, IL-7, IL9 and IL-15) (133). IL-4 (as well as IL-13), target the C epsilon gene for switch recombination (129) and (134). This is signal 1 of the process. At this stage the B cell receives both signals required for IgE switching: IL-4 triggers ε germ-line transcription, thereby targeting the ε gene region for recombination. Cross-linking of CD40 with CD40L activates DNA recombination to the targeted ε switch region, leading to IgE isotype switching and IgE secretion (124). It has become clear that in the absence of IL-4 no substantial IgE synthesis is possible, although some murine knockout models are somewhat leaky in this respect (135). IL-4 appears to act by selectively stimulating germ line transcription at the CHγ1 locus making the CHγ1 gene accessible to the action of a putative switch recombination enzyme (70). IL-4 also induces the appearance of germ-line CHε RNA and stimulates CHε rearrangement and IgE secretion "*in vitro*" (136).

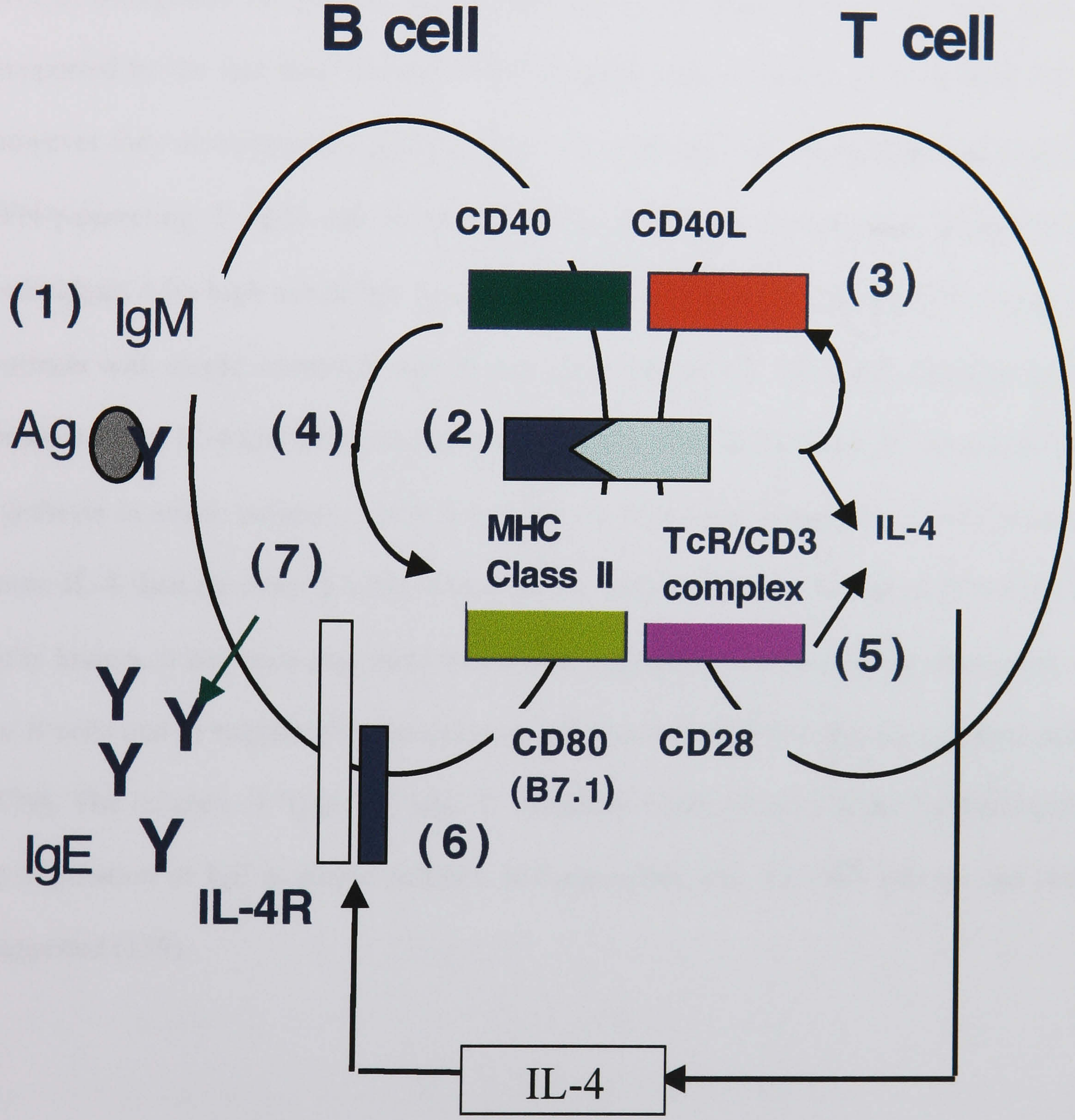


Figure 1.6 : T – B cell interactions in IgE switching.

1.9.1 IL-4/IFN-γ balance

The importance of IL-4 in IgE switching in humans, was first demonstrated by (109) and (110). The nature of IL-4 as a "type 2" cytokine has also been confirmed (119). The Th1/Th2 hypothesis was taken further by proving that "type 1" cytokines, such as IFN-γ, will downregulate the process of IgE production "in vitro" (110). This was further supported by the fact that helminth infected individuals can produce some IL-4 (137), however they also increased IFN-γ production reflected both by expanded numbers of IFN-γ-secreting T cells and increased IFN-γ in culture supernatants compared to individuals with high serum IgE levels. A reduced production of IFN-γ in the serum of patients with atopic dermatitis was also observed. However, a disturbed balance in the production of IL-4 and IFN-γ is unlikely to be the only explanation for increased IgE synthesis in atopic patients, and it is not known why allergen-specific T cells produce more IL-4 than do other T cells. Although the exact inhibitory action of IFN-γ is not fully known, it has been suggested that it acts indirectly, by blocking the effect of IL-4 on B cells and by suppressing the ongoing IgE production by the already switched cells (138). The balance of "type 1"/"type 2" cytokines might be the key for explaining the dysregulation of IgE in atopic patients, and a possible link with this balance has been suggested (139).

1.9.2 IL-13

IL-13 can cause IgE switching in human (not mouse) B cells activated by CD4+ or CD8+ T cells, but not to the same extent as IL-4 (140). IL-4 and IL-13 are structurally and functionally related and are most likely the product of gene duplication. IL-13 did not have any additive or synergistic effects on IL-4-induced IgE synthesis when both cytokines were added at saturating concentrations, suggesting similarities of the signaling pathways of these two cytokines (111). This was further supported by the observation that mAbs specific for the IL-4R α-chain were shown to block the biological functions of both IL-4 and IL-13, indicating that the IL-4R α-chain is a common component of both IL-4R and IL-13R complexes (141). The main difference between the biological functions of IL-4 and IL-13 is that the latter can not drive Th2 cell-type differentiation (142). IL-13 was found to have an indirect mode of action regulating IgE secretion, as it was found that it inhibits production of IFN-γ and IL-12 by monocytes (111).

1.9.3 IL-2

When B-cell cultures were stimulated with IL-4 in the presence of autologous T cells, a significant and dose-related increase of IgE synthesis was found. Such an increase was abolished by the addition of anti-IL-2 antibody. Further experiments showed that IL-2, even in the presence of IL-4, was unable to switch B cells to IgE, in the absence of T cells (143), probably due to the absence of the important CD40L. However, its presence

in culture was apparently important for the IL-4-dependent "in vitro" IgE synthesis. The potentiating action of IL-2 was indirect as it caused T cell proliferation which in turn secreted cytokines (such as IL-4, secreted by Th2 cells), that would cause B-cell switching to IgE. IL-2 has been shown to have variable effects on B cells which have been proven to be direct as B cells possess IL-2R (144). Experiments with IL-2 showed that IL-4-induced IgE synthesis "in vitro", by B cells from BALB/c or nude mice, was inhibited when rIL-2 was added to the culture, in the absence of T cells (145). This inhibition, which is expected as IL-2 is a "type 1" cytokine, was more pronounced when the cytokine was added at day 0. Addition of anti-IL-2 antibody reduced the inhibition, whereas addition of anti-IFN-γ had no action on the reduction produced by IL-2, proving that the inhibition of IgE by the rIL-2 was due to this cytokine. The suppression of IgE and IgG1 by IL-2 is probably different from that produced by IFN-γ.

1.9.4 IL-6

IL-6 had a synergistic effect when added with IL-4 to B-cell cultures, increasing the levels of IgE produced (143). Anti-IL-6 was found to have a partial inhibitory effect on IgE synthesis. In addition, IL-6 was able to enhance spontaneous IgE production by B cells from patients with severe atopy, suggesting that it may act on cells already producing IgE, probably amplifying the secretion of this Ig class, as it does for other Igs too. Punnonen and de Vries, (1994), also demonstrated a synergistic effect of IL-6 on IL-4 in B-cell proliferation, IgG4 and IgE production by foetal immature B cells and highly purified CD19+ tonsillar B cells (146).

1.9.5 IL-5

In several experimental systems, IL-5 has been shown to enhance secretion of IgG1 and IgE from B cells stimulated with IL-4. For example. IL-5 induces secretion of IgE of human PBL stimulated with IL-4 (126). Results from another study on IL-5 concluded that it can provide a second signal necessary for isotype switching to IgG1 and IgE (147). However. this effect of IL-5 is dependent on an initial B-cell activation stimulus. such as cross-linking surface Ig. or contact with plasma membranes, isolated from activated T cells. Thus, IL-5 cannot replace completely the signals provided by the B-cell contact with T cells.

1.9.6 IL-9

IL-9 can have a direct potentiating effect on IL-4-dependent IgE switching of murine B cells "in vitro". An indirect effect of IL-9 was suggested, as it was found to cause an increase in the production of IL-6 by B lymphocytes (148).

1.9.7 IL-7

IL-7 enhances Ig recombinase gene activity in human progenitor B cells and consequently may affect VDJ recombination (149). Jeannin *et al.,* (1998), showed that IL-7 synergises with IL-4 to induce human IgE and IgG4 production in a T-cell–dependent fashion (150). This effect is mediated at least in part through an increase

of both cell surface markers (such as CD23) and other cytokines such as IL-4, IL-6, IL-9 and IL-13.

1.9.8 IL-10

IL-10 is a cytokine produced by several cell types after activation, including the TH0, Th1 and Th2 CD4+ T cells, a proportion of CD8+ cells, B cells and monocytes (151). IL-10 prevents Ag-specific activation and proliferation of T cells by reducing the Ag-presenting capacity of monocytes, which is associated with a strong down-regulation of class II MHC molecules on these cells (152). In addition, IL-10 inhibits the secretion of pro-inflammatory cytokines and is an important suppressor factor for immunoproliferative and inflammatory responses IL-10 is also reported to inhibit spontaneous IL-4 induced IgE production by PBMCs (20 ng/ml => 2.3 ng/ml of IgE) in a dose-dependent fashion (153). The inhibitory effects were specific for IL-10, because they were completely neutralized by anti-IL-10 mAb (153).

1.9.9 IL-12

IL-12 promotes cell-mediated Th1 responses followed by an increase in the secretion of IFN-γ that downregulates IgE production by human PBMCs (154). The most potent effects of IL-12 occur when it synergises with IL-18. The latter can be synthesised by macrophages and DCs, and in combination with IL-12, can induce an even stronger Th1-type response accompanied by production of IFN-γ (155). IL-18 was shown to have no

effect on the IL-4-dependent IgE secretion when cultured with BALB/c mouse B cells (156). Combination of IL-12 and IL-18 is known to downregulate the production of IgE by human B cells via the induction of IFN-γ secretion by atopic PBMCs and human NK cells (157), (158) and (159).

1.9.10 TGF-β

It is also well established that TGF-β is an IgA switch factor for both murine and human B cells. The inhibitory effect of this cytokine on the IL-4-dependent IgE switching can be brought about directly (inhibition at the molecular level) and indirectly (preferential switching to the IgA isotype) (160).

1.10 Other factors affecting IgE switching.

1.10.1 Chemokines.

Chemokines can act on various cell types, including neutrophils, eosinophils, basophils, monocytes, myeloid progenitors, T and B cells (161). A study carried out by Kimata et al., (1996), demonstrated that RANTES and MIP-1α, both members of the β-chemokine subfamily, enhanced IgE and IgG4 production induced by IL-4 plus anti-CD40 m Ab in a dose-dependant fashion (162). In contrast, no other chemokines of the β-subfamily had

any effect on the production of IgG4 and IgE, at any concentrations. Enhancement of IgE production by RANTES and MIP-1α was specific, as there was inhibition of this production after the addition of anti-RANTES or anti-MIP-1α antibodies respectively. The enhancement was caused by stimulation of Ig production and not by proliferation of IgG4+ and IgE+ B cells, since RANTES and MIP-1α have no proliferating effects on B cells.

The fact that IL-4 inhibits IL-8 production by monocytes lead to the study of the effect of IL-8 on IL-4-induced IgE production. It was demonstrated that IL-8, a member of the α-subfamily of chemokines, selectively inhibited IgE production in MNC stimulated with IL-4 (163). Inhibition by IL-8 was specific since it could be blocked by anti IL-8 antibodies but not by control mouse IgG1. IL-8 also selectively inhibited IgE production by B cells stimulated with IL-4 and anti-CD40 antibody (163). Kinetic experiments showed that IL-8 had to be added at the initiation of the culture. Delayed addition of IL-8 after 1 day of culture had no effect. These results indicate that IL-8 inhibits the early activation step during IL-4 stimulation, and that the inhibition is not due to cytotoxicity. Preliminary work had also shown that IL-8 inhibits proliferation of human B-cell lines stimulated with IL-4 (164).

1.10.2 CD30.

The importance of CD40 ligation in providing the secondary signal for IgE switching, (as well as for B cell survival), is widely accepted. However, the signaling pathway through CD40 does not seem to be identical to that induced by activated CD4+ T cells

suggesting that additional membrane bound molecules are involved in the induction of B-cell proliferation, isotype switching and differentiation (165). Cerruti et al., used a monoclonal model of germinal centre maturation (CL-01 B cells), to investigate the role of CD30 in human B-cell differentiation (166). The results suggested that CD30 critically regulates the CD40-mediated differentiation of non-antigen selected human B cells. Switching was hampered by CD30 co-engagement, possibly through interference with the CD40-mediated NF-kappaB-dependent transcriptional activation of down-stream C(H) genes (166).

1.10.3 CD23/CD21

CD23 is a type II membrane glycoprotein containing four highly conserved and two partially conserved cysteines with C-type lectins (167). The CD23 molecule is expressed on a wide variety of cells, including B cells, T cells, a subset of thymic epithelial cells, follicular dendritic cells, Langerhans cells, monocytes eosinophils, platelets and EBV-containing nasopharyngeal carcinoma cells (168). IL-4 and IL-13 increase CD23 expression. IFN-γ, which inhibits IL-4 dependent-IgE switching of B cells, was also found to inhibit the IL-4-mediated expression of CD23 on B cells (169). sCD23 has been shown to augment ongoing spontaneous IgE production by B cells obtained from atopic individuals, and to act synergistically with sub-optimal concentrations of IL-4 to induce IgE production by normal human B cells (170). The importance of CD23 or its soluble fragments in the synthesis of human IgE "in vitro" was highlighted by the demonstration that certain anti-CD23 mAbs blocked IL-4-induced IgE production by normal B cells as

well as B cells obtained from atopic patients (96). It was mentioned earlier that the production of IgE "in vivo" requires direct contact between B and T cells. The potentiated action of CD23 in IgE secretion involves this T/B cell interaction, something that was proven when addition of anti-CD23 mAbs in culture inhibited the formation of T/B conjugates (171). Studies using inhibitory anti-CD21 Abs, as well as the binding of CD23-liposomes to recombinant CD21-transfected cells have revealed that CD23 binds to a subtype of CD21, a molecule that had previously been identified as a receptor for EBV and as complement receptor-2. CD21 is expressed on B cells, follicular dendritic cells and on T cells. Anti-CD21 Abs were found to decrease T/B cell interactions (just like anti-CD23 Abs), something that was expected as CD23/CD21 pairing has a role in T/B cell interaction. Thus, CD21 appears to be another molecule involved in the formation of T/B cell conjugates. Molecular analysis revealed that triggering of CD21 increased the IL-4-dependent germline ε transcription levels and had a synergistic effect on the expression of ε transcript induced by T cells (172). Based on the observation that triggering of CD21 by either anti-CD21 Abs or by sCD23 enhances IgE production both in T-cell independent and dependent systems and based on the reported induction of CD23 on T cells by IL-4 and allergen, one can speculate that in allergic individuals, T-cell associated CD23 may interact with B-cell associated CD21 leading to an increase in IgE production (173). Moreover, the interaction between CD23/CD21 controls IgE production more selectively than CD40/CD40L, as CD23 was shown to modulate the Ag-specific IgE response in an isotype-selective manner "in vivo" (174). CD23, also known as FcεRII can bind IgE enhancing most antibody immune responses. The IgE-mediated enhancement is completely inhibited by mAbs specific for CD23 (175). "In

vitro". CD23 exerts its effects both as cell bound receptor and in a soluble form. Murine sCD23 was in early reports claimed not to bind IgE, but later data, proved that it can do so but with 100-fold lower affinity than the receptor form of CD23 (176). The only receptor reported to interact with human sCD23 was CR2/CD21 (177). Monoclonal Abs against murine CD21 or of soluble rCD21 almost completely inhibit the antibody response to conventional antigens (178). However, treatment of mice with anti-CD21 before immunization with IgE and antigen had no inhibitory effect. thus arguing against the possibility that sCD23, in complex with Ag and IgE, exerts an effect via CD21. Production of sCD23 is dependent on the proteolytic cleavage of membrane CD23, but the proteases involved in this process remain largely unknown, although recent evidence suggests that the main enzyme involved is a zinc-dependent metalloproteinase (179). Inhibition of the enzyme responsible for cleaving mCD23 would have a negative regulatory effect on IgE production. The use of GI129471 (a broad spectrum zinc-metalloproteinase inhibitor). indeed inhibited the production of sCD23 reducing the IL-4-dependent IgE secretion in purified B-cell cultures, after 14 day incubation. Such a profile of activity may be useful in the treatment of allergic diseases, especially in light of evidence suggesting that sCD23 may play a further role in allergic hypersensitivity reactions by directly inducing histamine release by human mast cells (180).

1.10.4 CD28 / B7.1+B7.2

In a study carried out by Life *et al.*, it was shown that anti-CD28 mAbs produce a dose-dependent inhibition of IgE, but not IgG synthesis, by tonsillar B cells when driven by

an allergen specific T-cell clone in the presence of IL-4 (181). When a T-cell clone and tonsillar B cells were co-cultured with IL-4 under the conditions used to induce IgE synthesis, there was a progressive increase in the expression of CD28 on the T cells, while the expression of another T-cell marker, CD45RB, was not affected. Co-incubation of T and B-cell cultures with a dialyzed anti-CD28 mAb for 14 days resulted in a very strong and dose-dependent inhibition of IgE synthesis which was accompanied by a decrease in cell aggregate formation. The effect on IgG synthesis was considerably less marked and may be explained by a specific reduction of IgG4 isotype, the production of which is both IL-4 and T-cell dependent (182). Anti-CD28 mAbs inhibited the conjugate formation between T and B cells, which as previously mentioned is required for IgE switching. This is probably the inhibiting mode of action demonstrated by the anti-CD28 mAbs. CD28 binds with B7.1 and B7.2 which can be found in many cell populations such as B cells. Its interesting to note that in the study of Life *et al.*, addition of anti-B7.1 antibodies in the T/B cell co-cultures did not have any effect on the synthesis of IgE (181). This was contradicted by the study of Jeannin et al., (1997), which showed that the anti-B7.2 antibody, IT2.2, could potentiate IgE and IgG4 production by human B cells "in vitro", in the presence of IL-4 and anti-CD40 (183). CD28 expression on T cells increased in the presence of IL-4, as well as the levels of B7.1 and B7.2 on the B cells. This fact, together with the knowledge that IL-4 would activate Th2 cells, provides us with further evidence about the potentiating effect of IL-4 on IgE switching and synthesis by the B cells.

1.10.5 ICAM-1/LFA-1

Recently, signaling through ICAM-1 has been shown to complement the help provided by CD40 ligation to increase Ig synthesis by B cells and anti-LFA-1 and ICAM-1 mAbs can inhibit T-cell driven Ig production "in vitro" (184).

1.10.6 mTNF-α

In another study, by Aversa *et al.,* (1993). it was demonstrated that the 26-kD mTNF-α expressed on activated CD4+ T-cell clones provides a co-stimulatory signal required for IL-4-dependent Ig production. including IgG4 and IgE synthesis by B cells . mAbs against both mTNF-α and the p55 TNF receptor (TNFR) inhibited Ig production, indicating that in addition to CD40L-CD40 signaling, interactions between mTNF-α and its putative ligand, p55 TNFR, are involved in the induction of B-cell activation by activated CD4+ Th cells (185). Anti-TNF-α mAbs did not block Ig production induced by anti-CD40 mAbs and IL-4 in the absence of T cells, indicating that inhibition occurred at the Th cell and not at the B-cell level. In addition, the observation that anti-TNF-α mAbs effectively blocked Ig production in cultures where intact CD4+ T-cell clones were replaced by their plasma membranes, is consistent with the idea that mTNF-α expressed on activated T-cell clones, rather than soluble TNF-α produced from these clones, was the target of the anti-TNF-α mAbs. This notion was further supported by the

finding that pre-treatment of the T-cell plasma membranes with anti-TNF-α mAbs decreased their capacity to induce B cells to produce Ig (185).

1.10.7 LFA-3 (CD58)/CD2

LFA-3 (CD58), is a cell-adhesion molecule distributed on cells of different tissues and present on most blood cells. Its natural ligand is CD2 present on T cells (186). Studies investigating the role of CD58/CD2 interactions in human B-cell differentiation have shown that mAbs to both CD58 and CD2 inhibited T-dependent B-cell responses by interfering with cell-cell interaction (187). In a study carried out by Diaz-Sanchez *et al.*, (1994), it was shown that ligation of B-cell surface CD58 can provide IL-4 stimulated B cells with a second signal to induce IgE production in the absence of T cells or anti-CD40 (188). Murine T hybridomas transfected with CD2 could induce IgE production by purified B cells stimulated with IL-4, suggesting that the CD58/CD2 interaction can provide the stimulus for class switching and subsequent production of IgE. The role of CD58 in IgE switching is distinct from that of CD40 stimulation, showing that adhesion molecules may play a vital role in the regulation of IgE switching. In the experiments by Diaz-Sanchez *et al.*, no other isotypes were produced by the purified B cells in the culture (188). Whether the IgE-isotype specificity was due to the CD58 surface molecule or to the presence of IL-4 was not determined. The fact that the signal for IgE synthesis provided by CD58, is independent of the CD40 binding, was confirmed by addition of a CD40-Fc fusion protein, which caused 65% inhibition of CD40-driven IgE production, but did not cause a decrease in the CD58-driven IgE production. Furthermore, human

CD2. the natural ligand of CD58. when expressed on murine hybridoma cells. induced an IgE response in the absence of CD40. The addition of both anti CD58 and anti-CD40 Abs to B cells cultured with IL-4 did not result in synergy, no co-stimulatory effects on IgE production were observed. and CD40 always gave greater IgE responses. These findings suggest that CD58 and CD40. although differing in their surface receptors. may share intracellular elements in IgE induction as has been described for human IL-4 and IL-13 (133).

1.10.8 CD59.

A second ligand has been described for CD2; this new ligand, CD59, is involved in T-cell adhesion (189). Whether it too can play a role in IgE induction has yet to be determined. Finally, human and murine NK cells have been shown to play a role in IgE production, thought to involve CD23 (190). Because NK cells express CD2, this might be an additional ligand by which they can modulate IgE switching and production by B cells.

1.10.9 Other biological molecules.

Apart from the various cytokines and the cell surface molecules that can regulate IgE switching there are other factors that can also affect this process. For example, if B cells are already primed with a certain antigen, then the nature of the antigen will dictate whether the B cells can switch to IgE synthesis in the presence of IL-4. In such a study,

Snapper et al., (1991). showed that IL-4 containing CD4+ T-cell supernatant stimulated large amounts of IgG1 and IgE from LPS-activated B cells, but failed to stimulate detectable levels of IgE with αδ-dex-activated cells, despite inducing high levels of IgM and IgG1 (191).

Other biological molecules that can modulate IgE switching are the retinoids. Their role in the process was analyzed by Tokuyama and Tokuyama (1996). using LPS-stimulated murine B cells were cultured with IL-4 and IL-5 which enhanced IgE production. This study found that the synergistic effect of IL-5 in IgE synthesis could be attenuated by the addition of retinoids at a concentration of 1nM (192).

Another group of molecules which contribute to B-cell differentiation and hence, IgE switching, are the arachidonic acid (AA) metabolites (193). To evaluate the exact role of AA metabolites in the regulation of IgE production. Punnonen and co-workers added indomethacin, an inhibitor of the cylcooxygenase pathway, or NDGA, an inhibitor of the lipooxygenase pathway, to IL-4-treated cultures. Both indomethacin and NDGA inhibited IL-4 induced IgG4 and IgE production to the same extent (193). The inhibitory action of the AA metabolites is not fully elucidated but may involve a combination of many molecular factors.

Prostaglandins (PG) of the E series are generally known to suppress immune responses. However studies involving the PGs, carried out by Roper *et al.*, found that PGE synergized with IL-4 to induce IgE and IgG1 production in LPS-stimulated murine B lymphocytes (194). PGE1 and 2 significantly increased IgG1 and IgE production (up to 26-fold) at all concentrations of IL-4 tested (194). In addition to its effects on IgG1 and IgE, PGE also causes a significant decrease in IgM and IgG3 synthesis, suggesting that PGE may promote IL-4-induced class switching. PGE usually mediates its effects by

increasing the levels of cAMP. This was confirmed from the fact that other agents that can increase the levels of cAMP, such as cholera toxin, can also enhance the production of IgG1 and IgE "in vitro", while diminishing that of IgG3 and IgM. A further study by Roper et al., demonstrated that PGE increases the number of splenic B cells secreting IgE in culture, and that PGE synergizes with IL-4 and LPS in the IgE switching process of B cells (195). It was also found that PGE acts on the level of transcription as it aids IL-4 to induce ε germline transcripts. Thus, PGE may play a role in atopy "in vivo", were PGE-secreting cells, such as macrophages, follicular dendritic cells and fibroblasts can promote IgE synthesis (195).

Finally, Hasbold and co-workers, showed that recombinant CD40L and IL-4 are sufficient to induce switching to both the IgG1 and IgE isotype but also each of the switching events is related to division cycle number (196). This is consistent with the study carried out by Lundgren et al., who proved that B-cell switching requires DNA replication (197). This principle is further supported by an "in vivo" study initially done by Kind and Malloy, showing that Ag-specific IgG+ cells usually appear in the blood 3-6 days after immunization, becoming more numerous with time, while the IgE-secreting cells appear in the circulation after day 6 (198). The relationship between division and isotype expression was examined by Hasbold *et al.*, by stimulating B cells with membranes of insect cells infected with CD40L recombinant baculovirus and recombinant IL-4. From this experiment it was found that IgM, IgD and IgG1 expression changed in a cell-division manner, and most importantly that IgE expression was also independent of culture time but more related to the division cycle number (196).

1.11 T CELLS.

T-lymphocytes are antigen-specific cells of the immune system that recognise antigen by means of cell surface antigen receptor (TCR). Based on the expression of co-receptor molecules, T cells can be subdivided into CD4+ and CD8+ T cells. CD4+ T cells are mainly seen as helper T cells involved in both humoral and inflammatory immune responses while CD8+ T cells are traditionally seen as cytotoxic cells which specialise in killing virus infected cells. In addition, the existence of a distinct subset of T cells expressing γδ as opposed to αβ TCR chains, has been recently established (199) and (200). These γδ T cells are present in the lymphoid organs but are especially abundant in epithelial tissues where they are thought to be involved in innate immunity (201).

1.11.1 Functional Subsets of CD4 T cells.

The heterogeneity of CD4 cells based on cytokine secretion patterns was first described more than a decade ago (202). Two distinct cytokine secretion patterns were first recognised among differentiated mouse CD4 T cells. Th1 cells secrete interleukin-2 (IL-2), interferon-γ (IFN-γ) and lymphotoxin (LT), whereas Th2 cells secrete IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13 (202). Human CD4 T cells display similar cytokine secreting phenotypes, although the synthesis of IL-10 is not tightly restricted to the Th2 subset (203). Some cytokines, such as IL-3 and GM-CSF, are secreted by both Th1 and Th2 cells (204). Th0 cells expressing both type 1 and type 2 cytkines have been described (205) and (206) in human and mouse systems (207) and (208). Whether Th0 cells are the

only precursors for Th1 and Th2 cells (209), (210) or represent a separate, stably differentiated phenotype (211), (212) and (213) is still unclear. Th0 cells could play a role in eliminating pathogens in situations were a balance of cell mediated and humoral responses would ensure both eradication of the pathogen and minimal immunopathology.

These subsets are not an *in vitro* phenomenon as Th1 and Th2 cells develop during an immune response and are present at sites of inflammation (214) and (215). Although many freshly isolated populations secrete a non-restricted, Th0 like panel of cytokines, chronic inflammatory conditions usually result in highly polarised Th1/Th2 responses. This is probably achieved through the cross-inhibitory effects of the cytokines they produce. At the single cell level, among short-term T cell populations, IL-4 and IL-5 are often co-expressed whereas IL-4 and IFN-γ are mutually exclusive (216), (217), (218) and (219). However, single cell cytokine expression also indicates that patterns other than the extreme Th1 and Th2 exist: for example the co-expression of IL-10 and IFN-γ (216). In addition, in some systems, CD4 clones show a random distribution of type 1 and type 2 cytokines, raising the question of the validity of the Th1/Th2 paradigm (206) and (207). However, most clone data supports an inverse correlation of IL-4 and IFN-γ and positive association between IL-4 and IL-5 (206).

The distinct cytokine profiles of Th1 and Th2 cells are closely associated with their functional capabilities. Th1 cells are primarily involved in cell-mediated inflammatory reactions, whereas Th2 cells play a major role in humoral and allergic responses.

1.11.2 Functional Subsets of CD8 T Cells.

Historically, CD8 cells have been regarded as a homogeneous population of CD4-dependent cytotoxic cells producing IFN-γ, directed against viral infections and intracellular pathogens. The role of CD8+ T cells in immune suppression has been identified. These cells got known as suppressor T cells (220), (221), (222) and (223). Recently, CD8 T cells have been found to secrete a wide variety of cytokines indicating a potential role in immune regulation.

A number of groups have shown that both human and mouse CD8 T cells are able to produce IL-4 when stimulated with anti-CD3 or mitogens (224) and (225), especially when primed *in vitro* in the presence of IL-2 and IL-4 (226) and (227). It was also observed that freshly isolated peripheral blood CD8 T cells have the potential to make IL-4 at similar if not greater levels than those produced by comparable CD4 T cells (228).

CD8 T cells secreting type 2 cytokines have also been isolated in a variety of diseases indicating that such cells are more than an *in vitro* phenomenon. Salgame et al. reported that some of the *M leprae*-specific CD8 clones generated from the patients with Lepromatous leprosy made IL-4, IL-5 and IFN-γ but not IL-6 or IL-10 (221). These clones were termed type 2 clones or suppressor clones. However, they were atypical as they were apparently MHC class II restricted while IL-6 and IL-10 are usually secreted by such clones (229). Th2-like CD8 T cells secreting IL-4 and/or IL-5 and no IFN-γ have also been found in HIV infected individuals (229). CD8 T cells producing high levels of IL-4 and IL-5, have also been isolated from lungs of asthmatic patients (237),

(258) and (230).

In the mouse, CD8 T cell subsets comparable to Th1 and Th2 have been generated from naive precursors following stimulation by antigen presenting cells in the presence of the polarising cytokines (231). Analogous to the CD4 nomenclature these subsets were termed Tc1 (secreting IL-2 and IFN-γ and Tc2 (secreting IL-4 and IL-5 and variably reduced amounts of IFN-γ). These results were confirmed by Sad and colleagues using cytokine primed alloantigen-stimulated CD8 T cells (232) and by Cronin and colleagues using a regimen of repeated stimulation without priming with exogenous cytokines (233). In human and rat, synthesis of IL-2, IL-6, IL-10 and IL-13 do not fit the Th1/Th2 paradigm as closely as in the mouse and such clear cut patterns of cytokine production are less readily observed (234). In addition to the human Tc2 clones from HIV patients such clones have also been generated from peripheral blood and peritoneum of healthy individuals (235) and the gingiva of patients with chronic adult periodontitis (236).

The studies mentioned above clearly demonstrate that human, murine and rat CD8 T cells have the potential to produce a much wider array of cytokines than was initially thought. Moreover, CD8 T cells appear to differentiate in a polarised fashion and can be divided into subsets analogous to those described for CD4 T cells. These subsets are alternatively termed Tc1; and Tc2.

Unlike CD4 T cells, which readily differentiate into either Th1 or Th2 cells, naive CD8 T cells show a strong bias for differentiation into Tc1 cells (237), (238) and (239). In both cases cytokines play the major role in driving the differentiation process of these cells. Two early studies indicated that IL-4 was a strong inducing factor for IL-4

secretion by CD8 cells (215). The effect of different cytokines on the differentiation of naive CD8 precursors has subsequently been investigated further. In the mouse, results show that as for CD4 T cells, IL-12 and IFN-γ promote Type 1 and that IL-4, especially in the presence of anti-IFN-γ, promotes type 2 cells (231) and (232). Furthermore, in the rat (234) and mouse (232). IL-4 was found to inhibit the formation of IL-2 producing CD8 cells and promote IL-4 and IL-5 expression. Similarly, neonatal (naive) human CD8 T cells produced IL-4 and IL-5 after exposure to large amounts of IL-4 and IL-2 while IL-12 enhanced the capacity of primed cells to produce IFN-γ (240).

Very little is known about the molecular mechanisms of CD8 polarisation, and they may be similar or distinct from those observed in CD4 T cells. It appears that the transcription factor T-bet plays a similar role in Tc1 as in Th1 cells. The role of GATA-3 and c-maf in Tc2 is not clear, but the dependence of IL-4 production in Tc2 cells on STAT-6 has been demonstrated (241) and (242). One of the lineage specific differences that has been observed is the requirement for STAT-4 activation for IFN-γ production (243). It appears that while both CD4 and CD8 T cells require STAT-4 in IL-12 /IL-18 mediated induction of IFN-γ, only CD4 T cells require STAT-4 for TCR induced IFN-γ production.

1.11.3 CD8 T cell function

Traditionally, CD4 T cells have been regarded as helper cells that assist B cell antibody production, and CD8 T cells as cytotoxic cells that kill virally infected cells. This view

of the division of labour of these two T cell subsets is, in many ways, still true today. There are, however, a number of exceptions and CD4 T cells are now known to be capable of killing (244) and (245) while CD8 T cells may provide help for antibody responses (246), (247) and (248). The discovery of the Th1 and Th2 subsets of CD4 T cells had a huge impact on our understanding of immunology because the different cytokine profiles were closely associated with specific functions; i.e. Th1 cells were inflammatory T cells, while Th2 cells were helpers for antibody production. Since some CD8 T cells can produce potent immunoregulatory cytokines such as IL-4 , IL-10 (249) and TGF-ß (250), they may play distinct roles in both the induction and effector phases of the immune response which are considered below. However a correlation between cytokine profile and function has yet to be defined clearly for CD8 T cells.

Apart from their "in vivo" cytotoxic ability, CD8 T cells are known to recruit and activate inflammatory cells such as: macrophages, NK cells, neutrophils, eosinophils or mast cells/basophils in order to eliminate infection. CD8 T cells are strong inducers of DTH, a cell mediated inflammatory reaction. IFN-γ, produced by CD8 T cells, is present at the site of inflammation and is chemotactic to at least some of the cells (macrophages, neutrophils) that migrate to the site of inflammation, causing DTH swelling. Other cytokines or chemokines such as TNF-α IL-8 and MCP-1 (251) are also involved in recruiting neutrophils and monocytes. Interestingly, Tc2 cells, which do not produce IFN-γ can also induce substantial DTH (252).

CD8 T cells have been also found to regulate CD4 development. CD8 T cells can alter the balance of Th1/Th2 responses *in vivo* (253) and (254) by influencing the development of IL-4 or IFN-γ secreting CD4 cells (255) and (256).

T-cell mediated suppression is another CD8 T cell function. Suppression may be mediated by the production of particular cytokines such as IL-4, TGF-ß or IL-10, or by killing specific effector target cells (221). (222) (257) (223).

1.11.4 Tr and Th3 cells.

Recently it has been suggested that Th1 and Th2 may not be the only cytokine phenotypes of CD4 T cells. Many studies suggest that alternative regulatory populations exist, which may be somehow associated with, but distinct from Th2 cells (258) (259) and (260). Cells producing high amounts of TGF-β and variable levels of IL-4 and IL-10 have been isolated from mice orally tolerised to myelin basic protein (EAE- inducing agent) and named Th3 (261) and (262). These cells have been shown to suppress EAE. The induction of anergy by Th3 cells is thought to occur due to the downregulation of the antigen presenting process, possibly via the production of TGF-β (263). Similar regulatory T cells have been isolated from the pancreas of NOD mice and shown to suppress diabetes (264). Distinct CD4 T regulatory 1 (Tr1) cells characterised by high IL-10 production and low proliferative capabilities have also been described (265). These Tr1 cells inhibit antigen-specific responses in vitro and prevent T cell mediated disease (colitis) in vivo probably through the secretion of high levels of IL-10.

Regulatory CD4 T cell subsets can inhibit not only cell mediated immune responses that lead to autoimmunity (266), (267) (261) (268) and (265) but also inflammatory pathologies mediated by Th2 cells (258), (259) and (260). It appears that this

suppression can be attributed to TGF-β and /or IL-10 (in case of Tr1 cells) which are known to inhibit both Th1 and Th2 development (269), (270) and (271).

Both Th3 and Tr cells are thought to be involved in the development of T-cell anergy in both human and murine models, although the interaction between the two cell types is yet to be resolved (272).

1.12 B cell help.

The discovery that some CD8 T cells can secrete "helper" cytokines led to suggestions that these cells may be able to help B cells. Some cases of non-cytotoxic CD8 T cells that could provide help for Ig synthesis (via cell surface markers and/or secreted cytokines), have been reported, for example in HIV infection (273), and in CD8 T cell clones derived from skin window chamber cells from delayed type hypersensitivity (DTH) reactions elicited by either PPD or Candida, (274). CD8 T cells vigorously stimulated *in vitro* in the presence of high concentrations of IL-4 became $CD4^{-}CD8^{-}$, secreted large amounts of IL-4 and were capable of switching B cells to make IgE (275) and (227). This is confirmed by the finding that some CD8 clones express CD40L, a critical signal for antibody production (233) and can provide B cell help *in vitro* for switching to IgE (276), (273) and (277) and for IgG synthesis (246). It is therefore possible that CD8 T cells can contribute to antibody responses and that, as for CD4 cells, this is associated with reduced cell-mediated immunity. On the other hand, it is difficult to reconcile this function with the strong cytolytic activity of Tc2 cells (278). This is

also consistent with the antigen processing pathways used for MHC class I presentation. Thus, it is likely that Tc2 cells will express cytokines and surface molecules that may provide antigen non-specific help for neighbouring B cells, while providing efficient killing of infected B cells presenting appropriate Ag via MHC class I.

1.13 CD8 T cells and suppression of IgE

The ability of CD8 T cells to regulate the production of IgE is a well recognised example of T cell mediated suppression (279), (280), (281), (282) and (283). IgE inhibitory CD8 T cells have been generated following antigen inhalation (280) and (281) or parenteral immunisation (284) and (285). MHC class I restricted CD8 T cell clones specific for exogenous antigen have been shown to suppress IgE (286) (287). Furthermore, depletion of CD8 T cells in immunised animals enhances antigen-specific IL-4, and suppresses IFN-γ synthesis (254) suggesting that CD8 T cells switch the CD4 T cell response from Th2 to Th1.

The most obvious explanation for the suppressive ability of CD8 T cells, is the secretion of IFN-γ production during development of CD4 responses could inhibit the development of Th2 cells whilst favouring the development of Th1 cells (282). Alternatively CD8 T cell-derived IFN-γ could act directly on B cells to inhibit IgE synthesis and / or cytotoxic CD8 T cells could kill Ag-specific B cells or APCs. However the ability of ovalbumin (OVA)-specific CD8 T cell clones to inhibit IgE is unrelated to the levels of IFN-γ they produce in vitro or to their cytolitic activity (288). A study carried out by Thomas *et al.*, (2002), proved the ability of such clones to

stimulate IL-12 secretion by murine DCs, which in turn would favour the development of a Th1-type response over a Th2 one (289). This demonstrates a novel regulatory network through which CD8 T cells can inhibit allergic sensitisation in an IFN-γ-independent manner.

1.14 Work that has led up to this project.

Having established the importance of T cells in eliciting or suppressing IgE responses. the possible regulatory control of individual T-cell subsets on the levels of IgE has been studied both "*in vitro*" and "in vivo". Generation of Th1 and Th2 cells "in vivo", can skew immune responses accordingly depending on the antigen (290). and the antigen-presenting cell (291). Type 1 and type 2 immune responses are accompanied by an appropriate cytokine profile that can influence immune responses in both an autocrine and a paracrine fashion. In the case of IgE regulation, the latter immunoglobulin has been elevated during a dominant type 2 response (driven by IL-4) while the reverse has been observed in a type 1 immune response (292).

The existence of distinct CD8+ subpopulations in humans has been well established (221) and (273). These cells, classified into Tc1 and Tc2, were shown to secrete specific cytokine patterns that remained stable and fitted the Th1 / Th2 paradigm. This observation led to the study of IgE regulating potential of these CD8+ cell subsets. Such regulation can be indirect as the latter T cells play a role in the activation and differentiation of CD4+ T cells. This may be mediated through secreted products

(cytokines, chemokines) or by cell-cell interactions, giving rise to a relevant immune response which in turn would reflect on the levels of IgE secreted by B cells. Alternatively, secretion of type 1 or type 2 cytokines by Tc1 and Tc2 cells respectively might influence the relevant IgE responses directly.

Kemeny *et al.*, (1994), found that depletion of CD8+ cells in rats enhances IgE production and favours a Th2-type immune response when the animals have been previously immunised with ricin (293). This is probably due to the rise of IL-4 production by CD4+ T- cells although other immunoregulatory mechanisms might be also unmasked. Noble *et al.*, (1993), showed that ricin-sensitive CD8+ T cells might favour the development of a Th1 response which might explain the suppressive effect of these cells on IgE secretion (294). These observations led us to investigate the effects of different T-cell derived factors (including CD8+ soluble factors) on IgE synthesis "*in vitro*". The suppressive effects of human T-cells "*in vitro*" concerning IgE regulation were established (295). Erard *et al.*, (1993) and Vukmanovic-Stejic *et al.*, (2000), were able to generate human Tc1 and Tc2 clones "*in vitro*" that secreted distinct cytokine profiles (227) and (296). Hence, the basis for an "*in vitro*" study of the effects of secreted T cell factors on IgE regulation was set.

1.15 Aims of the thesis.

The study detailed in this manuscript investigated the ability of CD8+ and other secretory factors (e.g. cytokines) to stimulate human B cells to IgE ... class secretion "in vitro". The aims set and achieved in this work were the following:

1. **To confirm the effects of cytokines on IgE regulation and other immunoglobulin classes and subclasses.**

2. **To inhibit these effects with the appropriate blocking antibodies.**

3. **To test the effect of Tc1 and Tc2 soluble factors on IgE synthesis.**

4. **To identify modes of action of these factors that regulate IgE.**

CHAPTER 2: MATERIALS AND METHODS.

2.1 Tonsils.

Ethical committee approval to use human tonsils was obtained. Patients undergoing tonsillectomy gave informed consent for their tonsils to be used for research. Tonsils were collected from the operating theatres of Guy's Hospital in London on the day of the experiment. The donor had to be between the age range of 3-35 years of age and the tonsils (obtained fresh) had to be processed within the next 3 h. following the tonsillectomy.

2.2 Preparation of Sheep Red Blood Cells (SRBCs).

Sheep red blood cells in Alsever's solution were prepared (usually a day prior use), in order to deplete the T cells from the tonsillar cell suspension, by positive selection.

2.2.1 Reagents.

Sheep Red Blood Cells in Alsever's solution. (TCS Biosciences Ltd. Claydon, Buckingham, UK). 2-aminoethylisothiouronium hydrobromide (AET), RPMI-1640. (SIGMA. Pool, Dorset, UK). Phosphate Buffered Saline (PBS). (INVITROGEN Plc. Inchinnan Business Park, Paisley, UK).

2.2.2 Protocol.

1. 2-aminoethylisothiouronium hydrobromide (AET) was dissolved in distilled water at 0.5g/12.5 ml, the pH adjusted to 9.0 with 4M NaOH and sterile filtered.
2. 8 mls of sheep red blood cells (SRBC) in Alsever's solution were washed 5 times in sterile PBS, at 600g for 10 mins at 20°C.
3. After the final wash, 2ml of packed SRBC were incubated with 8 ml of AET solution for 20 mins at 37°C and washed 3 times with cold PBS at 400g for 10 mins.

4. The SRBC were resuspended in tissue culture medium (RPMI–1640/ 2mM L-Glutamine). AET treated SRBC could be stored at 4°C for a maximum of 7 days.

2.3 Isolation and purification of tonsilar or PBMC B cells by T-cell rosetting.

B cells were purified from the tonsillar cell suspension or from the PBMCs by the addition of AET-treated Sheep Red Blood Cells that would bind T cells creating a cell complex that was removed by the density gradient method using lymphoprep. The purity of the B-cells obtained was considered acceptable for our study.

2.3.1 Reagents.

Iscove's Modified Dulbecco's Medium (IMDM), L-Glutamine, human holo-transferrin, Ficoll Hypaque, NaCl powder, Penicillin/Streptomycin. (SIGMA. Poole, Dorset, UK).

AET-treated Sheep Red Blood Cells (previously prepared). F etal Calf Seum (FCS). (Harlan SERA-LAB. Belton, Loughborough, UK).

2.3.2 Solutions.

Complete Medium. (IMDM, 2μM L-Glutamine, 100 U/ml penicillin/streptomycin, 10% FCS and 50 μg/ml holo-transferrin).

Hypotonic sodium chloride (0.2%). (0.2 g. NaCl/100ml sterile H_2O).

Hypertonic sodium chloride (1.6%). (1.6 g. NaCl/100 ml sterile H_2O).

2.3.3 Protocol

1.Tonsils were obtained as described previously, cut into small fragments and suspended in complete medium.

2.Tonsillar tissue was forced through a 70μM cell strainer.

3. The cell suspension obtained was washed with complete medium for 10 min at 800g at 20°C and re-suspended in 20ml of complete medium, giving a final concentration of approximately $1x10^7$cells/ml.

4. 20 ml of the AET-treated packed SRBC were added to 10 ml of the tonsillar suspension, centrifuged for 2 min at 150g and incubated for 1 h at 4°C.

5. The rosetted cells were separated over 15mls of Ficoll Hypaque for 20 min. at 800g at 4°C.

6. B cells were collected from the interface. Remaining red blood cells in B cell preparation were lysed by adding equal amounts of 0.2% hypotonic sodium chloride solution followed by 1.6% hypertonic sodium chloride solution.

7. B cells were finally washed twice in complete medium for 10 min and centrifuged at 600g for 10 min. at 20°C and counted. The average yield was 15-20 x 10^7 B cells/tonsil.

8. Ficoll Hypaque was used to separate PBMCs from whole blood. 35ml of blood were added on 15ml of Ficoll Hypaque and centrifuged for 20min. at 800g and 4°C. Approximatelly, 10^6 PBMCs were obtained per 1ml of whole blood. The PBMC pool would go through the rosetting method in order to obtain B cells. 20% of the total PBMC number was found to be B cells.

2.4 Positive selection of tonsillar B cells by CD19+ Dynabeads.

This was an alternative way of purifying B cells from a tonsillar cell suspension. B cells had a higher purity compared to the T-cell rosetting method of purification although they yielded lower levels of IgE, which was related to the sensitivity of the cells and of the IgE secretion process.

2.4.1 Reagents.

CD19+ Dynabeads (M 450). DETACHaBEADS. (Dynal Ltd. Wirral. Merseyside. UK). Phosphate Buffered Saline (PBS). (INVITROGEN Plc. Inchinnan Business Park. Paisley. UK). RPMI-1640 (SIGMA. Pool. Dorset. UK). Foetal Calf Serum (FCS). (Harlan SERA-LAB. Belton. Loughborough. UK).

2.4.2 Protocol.

1. A tonsillar cell suspension was prepared as described before. Dynabeads M-450 CD19 (4x the number of target cells) were washed 3-5 times with PBS.
2. The dynabeads were added to the cell suspension to a final concentration of at least $1x10^7$ Dynabeads M-450/ml and incubated at 4°C with slow tilting and rotation for at least 1 h. (on magnet).
3. The cell suspension was then resuspended in 300µl of cell culture medium (eg.:RPMI1640/FCS) and 30µl of DETACHaBEAD added.
4. The cell suspension was further incubated for 45-60 min. at ambient temperature.
5. The released beads were then removed by placing the eppendorf in the dynal MPC for 2 min. and the cell suspension was then pippeted out of the eppendorf.
6. The process was repeated 2-3 times in order to obtain any residual cells. The total detached cells were then washed thoroughly by resuspending and centrifuging for 10 min. at 800g 2-3 times, in order to remove any DETACHaBEADS present.
7. The purified cells (>98% purity) were finally resuspended in 5 mls of culture media.

2.5 B-cell purity assessed by Flow Cytometry.

The purity level of B cells isolated by either T-cell rosetting or positive selection was assessed by surface staining followed by flow cytometry.

2.5.1 Reagents.

Phosphate Buffered Saline (PBS). (INVITROGEN Plc. Inchinnan Business Park. Paisley. UK). Bovine Serum Albumin (BSA). (SIGMA. Pool. Dorset. UK). PE-anti-CD19. FITC-anti-CD3. FITC-anti-CD14 conjugated antibodies. (Becton-Dickinson-PHARMINGEN. Cowley. Oxford. UK).

2.5.2 Protocol.

1. Approximately $5x10^5$ purified B cells were added per FACS tube and washed once with PBS + 0.5% BSA (Sigma) for 5 mins at 200g
2. Extracellular B cell staining was carried out adding 10µl PE – conjugated anti-CD19 antibody per FACS tube.
3. T-cell impurity was detected with 10µl FITC – conjugated anti-CD3 antibody while monocyte impurity was determined by adding 10µl FITC – conjugated anti-CD14 antibody.
4. After the monoclonal flow cytometry antibodies were added (double staining: anti-CD19 and anti-CD3 or anti-CD19 and anti-CD14), the FACS tubes were placed on ice and incubated for 20 mins (at 4^oC).
5. After the incubation period the cells were washed twice with PBS + 0.5% BSA (5 mins at 200g) and the purity was tested by flow cytometry.

2.6 Positive selection of CD4+/CD8+ T cells.

After a positive selection step in order to isolate the B cells from a tonsillar cell suspension, the CD4+/CD8+ T cells were isolated in the same manner by positive selection, using the relevant dynabeads.

2.6.1 Reagents.

CD4+/CD8+ Dynabeads , DETACHaBEADS. (Dynal Ltd. Wirral, Merseyside, UK). Phosphate Buffered Saline (PBS). (INVITROGEN Plc. Inchinnan Business Park, Paisley, UK). RPMI-1640 (SIGMA. Pool, Dorset, UK). Foetal Calf Seum (FCS). (Harlan SERA-LAB. Belton, Loughborough, UK).

2.6.2 Protocol.

1. CD8 cells were isolated first by positive selection using CD8 dynabeads, which The unbound cells were separated using a magnetic concentrator. Magnet was applied for 2 minutes and unbound cells aspirated.

2 The remaining dynabeads were gently resuspended in 1 ml of RPMI 1640/2% FCS and reapplied to the magnet for 30 seconds and the supernatant aspirated. This washing procedure was repeated 4 times in total and the beads resuspended in 100µl of RPMI 1640/2% FCS.

3 Bound CD8 cells were released by addition of the Detach-a-bead antibody.

4 Following incubation (with rotation) for 60 minutes at room temperature, the magnet was applied for 2 minutes and the released CD8 cells collected.

5 The dynabeads were then washed with 1 ml RPMI/2% FCS, the magnet reapplied for 30 seconds and the released CD8 cells collected as before. This procedure was repeated twice and all the released CD8 cells were pooled and washed twice by centrifugation at 200 g for 10 minutes to remove any residual Detach-a-bead solution.

6 The same procedure was carried out using CD4 dynabeads instead of CD8 in order to positively select the CD4+ T cells from the cell suspension.

2.7 CD4+/CD8+ T-cell purity assessed by flow cytometry.

Using flow cytometry, the purity of the CD4+ and CD8+ T cells was assessed in a very similar way compared to the assessment of B-cell purity described earlier on.

2.7.1 Reagents.

Phosphate Buffered Saline (PBS). (INVITROGEN Plc. Inchinnan Business Park, Paisley, UK). Bovine Serum Albumin (BSA). (SIGMA. Pool, Dorset, UK). PE-anti-CD4, FITC-anti-CD8 conjugated antibodies. (Becton-Dickinson-PHARMINGEN. Cowley, Oxford, UK).

2.7.2 Protocol.

1. Approximately $5x10^5$ purified T cells (CD4+ or CD8+) were added per FACS tube and washed once with PBS + 0.5% BSA (Sigma) for 5 mins at 200g.

2 Extracellular T cell double surface staining was carried out adding 10µl PE – conjugated anti-CD4 and FITC-conjugated anti-CD8 antibody per FACS tube.

3 After the monoclonal flow cytometry antibodies were added, the FACS tubes were placed in an ice bucket and incubated for 20 mins (at 4^oC).

4 After the incubation period the cells were washed twice with PBS + 0.5% BSA (5 mins at 200g) and the purity was tested by flow cytometry.

2.8 B-cell proliferation assay.

Tonsillar B cells purified by positive selection or T-cell rosetting were put into culture under various conditions and their proliferation was assessed by a standard proliferation assay.

2.8.1 Reagents.

^{3}H-Thymidine. (Amersham. Essex. UK).

2.8.2 Protocol.

1. Purified B cells were cultured at various conditions in flat-bottomed 96 well cell culture plates.
2. After 4 days the cells were pulsed with ^{3}H-Thymidine (0.5μCi/well) under sterile conditions.
3. The cells were then harvested for 18h and counted using a Matrix 96 Direct β-counter (Canberra Packard).
4. The results were displayed as counts per minute (cpm).

2.9 Optimisation of the IgE system.

In order to study the IgE production by tonsillar B cells, the latter were put into culture with IL-4 and anti-CD40 monoclonal antibody which have been proven to be essential for this process. These two antibodies were titrated in the cultures in order to find their optimum concentrations. Overall, switching human B cells to IgE secretion was proven to be a very sensitive process.

2.9.1 Reagents.

Iscove's Modified Dulbecco's Medium (IMDM), L-Glutamine, human holo-transferrin, Ficoll Hypaque, NaCl powder, Penicillin/Streptomycin, alkaline phsphatase conjugated anti-IgE (SIGMA. Pool, Dorset, UK). Foetal Calf Serum (FCS). (Harlan SERA-LAB. Belton, Loughborough, UK). AET-treated Sheep Red Blood Cells (previously prepared). Recombinant human IL-4 (Becton-Dickinson-PHARMINGEN. Cowley, Oxford, UK).

Anti-CD40 monoclonal antibody (Clone: G28.5), human anti-IgE monoclonal antibody (Clone: 577C1) (Gift by Dr. Darren Wheeler. AVENTIS. New Jersey, USA).

Human myeloma IgE. (Calbiochem. Beeston, Nottingham,UK).

2.9.2 Protocol

1. Tonsillar B cells were purified and isolated by T-cell rosetting, split into two groups and put into 96-well culture plates (10^5cells/well) in complete media (as described above).
2. One group of cells contained IL-4 at 20 ng/ml while the anti-CD40 monoclonal antibody was titrated in at a concentration range from 0-2 μg/ml.
3. The second group of B cells was similarly cultured but at a constant concentration of anti-CD40 monoclonal antibody (0.5 μg/ml), while the IL-4 was titrated between concentrations: 0-80 ng/ml.
4. Both sets of cultures were incubated for 10 days at 37°C.
5. Supernatants were then collected and the IgE levels were measured by an IgE ELISA (described later on).

2.10 Time-Course of IgE secretion.

In order to investigate the time course of IgE secretion by switched B cells "in vitro", the latter were put in culture under standard conditions of IL-4 and anti-CD40 and the supernatants were collected at set days so as to measure the levels of IgE being produced.

2.10.1 Reagents.

Iscove's Modified Dulbecco's Medium (IMDM), L-Glutamine, human holo-transferrin, Ficoll Hypaque, NaCl powder, Penicillin/Streptomycin, alkaline phosphatase conjugated anti-IgE (SIGMA, Pool, Dorset, UK), Foetal Calf Seum (FCS), (Harlan SERA-LAB, Belton, Loughborough, UK), AET-treated Sheep Red Blood Cells (previously prepared), Recombinant human IL-4 (Becton-Dickinson-PHARMINGEN, Cowley, Oxford, UK), Anti-CD40 monoclonal antibody (Clone: G28.5), human anti-IgE monoclonal antibody (Clone: 577C1) (Gift by Dr. Darren Wheeler, AVENTIS, New Jersey, USA), Human myeloma IgE, (Calbiochem, Beeston, Notingham,UK).

2.10.2 Protocol.

1. Tonsillar B cells were isolated by T-cell rosetting and cultured at 37°C with IL-4 (20 ng/ml) and anti-CD40 (0.5 μg/ml) in 96-well plates (10^5cells/well).
2. The supernatants were collected at days: 2, 4, 6, 10, 14 and 18, and
3. The IgE levels were measured by an IgE ELISA (as described below).

2.11 Cytokines and IgE production.

The effects of human recombinant cytokines on IgE production were studied. The cytokines: IL-2, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12, IFN-γ and TGF-β were titrated individually in the B-cell cultures. The levels of IgE in the supernatants were measured by a direct IgE sandwich ELISA after a 10 day incubation.

2.11.1 Reagents

Iscove's Modified Dulbecco's Medium (IMDM), L-Glutamine, human holo-transferrin, Ficoll Hypaque, NaCl powder, Penicillin/Streptomycin, alkaline phosphatase conjugated anti-IgE (SIGMA, Pool, Dorset, UK). Foetal Calf Seum (FCS). (Harlan SERA-LAB, Belton, Loughborough, UK). AET-treated Sheep Red Blood Cells (previously prepared). Recombinant human cytokines (Becton-Dickinson-PHARMINGEN, Cowley, Oxford, UK). Anti-CD40 monoclonal antibody (Clone: G28.5), human anti-IgE monoclonal antibody (Clone: 577C1) (Gift by Dr. Darren Wheeler, AVENTIS, New Jersey, USA). Human myeloma IgE. (Calbiochem, Beeston, Notingham,UK).

CD19+ dynabeads (M-450), DETACHaBEADS. (Dynal Ltd. Wirral, Merseyside, UK).

2.11.2 Protocol.

1. Tonsillar B cells were isolated by T-cell rosetting or positively selected by CD19+ dynabeads and cultured at 37°C with IL-4 (20 ng/ml) and anti-CD40 (0.5 μg/ml) in 96-well plates (10^5cells/well).
2. One additional exogenous cytokine was titrated in the cultures at day 0. The concentration range was: 0, 0.1, 1, 10, 100, 1000 Units/ml. Cytokines were reconstituted in sterile PBS and stord at –20°C.
3. After a 10 day incubation the supernatants were collected and the IgE levels were measured by a direct IgE sandwich ELISA (see below).

2.12 Tc supernatants.

The effect of Tc clone supernatants on IgE production was investigated. The Tc clones were stimulated a week prior to the collection of their supernatants with anti-CD3 and

anti-CD28 rather than PMA and ionomycin, excluding the chance of transferring the stimulus to the B cell cultures.

2.12.1 Reagents.

Iscove's Modified Dulbecco's Medium (IMDM), L-Glutamine, human holo-transferrin, Ficoll Hypaque, NaCl powder, Penicillin/Streptomycin, alkaline phosphatase conjugated anti-IgE (SIGMA, Pool, Dorset, UK). Foetal Calf Seum (FCS). (Harlan SERA-LAB, Belton, Loughborough, UK). AET-treated Sheep Red Blood Cells (previously prepared). Recombinant human IL-4 (Becton-Dickinson-PHARMINGEN, Cowley, Oxford, UK). Anti-CD40 monoclonal antibody (Clone: G28.5), human anti-IgE monoclonal antibody (Clone: 577C1) (Gift by Dr. Darren Wheeler, AVENTIS, New Jersey, USA). Human myeloma IgE. (Calbiochem, Beeston, Nottingham,UK).

Tc1 clones: A111, 2 and 5. **Tc2 clones:** B206, 1 and 4. (Kindly provided by Dr. Milica Vukmanovic-Stejic).

2.12.2 Protocol

1. Tonsillar B cells isolated by T-cell rosetting were put into culture (96-well plates) with IL-4 (20 ng/ml) and anti-CD40 (0.5 μg/ml), in complete media.
2. Tc clone supernatants: A111 and B206 were titrated in the 96-well plates with the B cells at standard conditions (with IL-4 and anti-CD40), producing a range of dilutions: 1/2, 1/10 and 1/100.
3. The cells were cultured for 10 days at 37°C.
4. Supernatants were then collected and the IgE levels were measured by a direct IgE sandwich ELISA (described below).

5. Tc clone supernatants: 1.4 (Tc2). 2 and 5 (Tc1) were added with the purified tonsillar B cells IL-4 and anti-CD40 in culture together with their relevant neutralising antibodies at their optimum concentrations (see below).
6. With the Tc2 clone supernatants anti-IL-4. anti-IL-6 and IL-10 were added to the culture.
7. With the Tc1 clone supernatants anti-IFN-γ was added.
8. B cells were also cultured in the absence of any exogenous IL-4 and/or supernatant.
9. After 10 days incubation at 37°C the B-cell supernatants were collected and the IgE levels were measured by a sandwich IgE ELISA (as described below).

2.13 IgE ELISA.

The IgE levels in cell supernatants were measured by a direct sandwich IgE ELISA.

2.13.1 Reagents

Alkaline phosphatase-anti-IgE polyclonal antibody, 1 p-nitrophenylphosphatase tablets, Bovine Serum Albumin (BSA), buffer chemicals. (SIGMA. Pool, Dorset, UK).

Human myeloma IgE. (Calbiochem. Beeston, Nottingham, UK).

Human anti-IgE monoclonal antibody (Clone: 577C1). (Gift by Dr. Darren Wheeler. AVENTIS. New Jersey, USA).

Phosphate Buffered Saline (PBS). (INVITROGEN Plc. Inchinnan Business Park, Paisley, UK). Tween-20. (Becton-Dickinson-PHARMINGEN. Cowley, Oxford. UK).

2.13.2 Buffers.

Coating Buffer: carbonate-bicarbonate buffer (0.1M, pH 9.6)

4.24g Na_2CO_3 and 5.04g $NaHCO_3$ was dissolved in 1 litre of distilled water. pH was checked and adjusted to 9.6 if necessary. Store at 4°C.

Wash Buffer: PBS/Tween

PBS: 5.79g $NaH_2PO_4xH_20$. 85g NaCl. 16.7g Na_2HPO_4 and 10g sodium azide were resuspended in 10l of distilled water. 5ml of Tween20 was added.

Substrate Buffer: Diethanolamine buffer(1l)

101mg of $MgCl_2.6H_20$ was added to 800ml distilled water and dissolved. 97ml of diethanolamine was added, with stirring, and when fully dissolved pH was adjusted to 9.8 with conc HCl. The volume is then adjusted to 1l with distilled water.

2.13.3 Protocol.

1. 96 well Maxisorp ELISA plates were coated with the primary antibody (anti-IgE clone: 577C1, at a concentration of 1.25 µg/ml in carbonate buffer (100µl/well).
2. The plates were then covered with seal and left in the fridge for overnight incubation.
3. After overnight incubation the plates were washed four times with wash buffer.
4. Each well was then blocked with 200µl of wash buffer and incubated for 30 mins at room temperature. Plates were then washed three times and 100µl of the standards and the B-cell supernatants were added per well.
5. The top standard concentration was 360 ng/ml while human serum was used each time as a quality control at 1/100 dilution.
6. The standards and supernatants were left to incubate for 2 h. at 37°C and then washed three more times.

7. Alkaline phosphatase labelled secondary antibody: anti-IgE. was added at 1/500 dilution, 100µl/well, and the plates were incubated for a further 1h. at 37°C.
8. The plates were finally washed five times and 1 p-Nitrophenyl phosphate solution was added at a concentration of 1mg/ml at 100µl/well.
9. The plates were incubated in the dark for at least $^1/_2$ h. in order to allow for the yellow colour to develop.
10. The OD values and their corresponding concentration levels were measured and calculated by the ELISA Macintosh programme: SOFTMAX (at 405nm wavelength).

2.14 IgA ELISA.

The levels of IgA in the cell supernatants were measured by direct IgA sandwich ELISA.

2.14.1 Reagents.

Human IgA ELISA Quantitation Kit. (BETHYL LAB. Montgomery, Texas, USA).

Phosphate Buffered Saline (PBS). (INVITROGEN Plc. Inchinnan Business Park, Paisley, UK). Tween-20. (Becton-Dickinson-PHARMINGEN. Cowley, Oxford, UK).

Buffer Chemicals (See buffer section). (SIGMA. Pool, Dorset, UK).

2.14.2 Protocol.

1. 96 well Maxisorb ELISA plates were coated with the primary antibody anti-IgA at a concentration of 1.25 µg/ml in carbonate buffer (100µl/well).
2. The plates were then covered with seal and left in the fridge for overnight incubation.

3. After overnight incubation the plates were washed four times with wash buffer.
4. Each well was then blocked with 200µl of wash buffer and incubated for 30 mins at room temperature. Plates were then washed three times and 100µl of the standards and the B-cell supernatants were added per well.
5. The top standard concentration was 360 ng/ml while human serum was used each time as a quality control at 1/100 dilution.
6. The standards and supernatants were left to incubate for 2 h. at 37°C and then washed three more times.
7. Horseradish peroxidase labelled secondary antibody: anti-IgA, was added at 1/500 dilution, 100µl/well, and the plates were incubated for a further 1h. at 37°C.
8. The plates were finally washed five times and 2,2-Azino-Bis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS) was added at a concentration of 1mg/ml at 100µl/well.
9. The plates were incubated in the dark for at least $^1/_2$ h. in order to allow for the green colour to develop.
10. The OD values and their corresponding concentration levels were measured and calculated by the ELISA Macintosh programme: SOFTMAX.

2.15 IgG1 ELISA.

The IgG1 levels in the cell supernatants were measured by a direct IgG1 sandwich ELISA.

2.15.1 Reagents.

Alkaline phosphatase-anti-IgG1 polyclonal antibody, 1 p-nitrophenylphosphatase tablets, Bovine Serum Albumin (BSA), buffer chemicals. (SIGMA. Pool, Dorset, UK).

Human myeloma IgG1. (Calbiochem. Beeston. Nottingham. UK). Human anti-IgG1 monoclonal antibody (Clone: MC003). (The Binding Site Ltd. Birmingham. UK). Phosphate Buffered Saline (PBS). (INVITROGEN Plc. Inchinnan Business Park. Paisley. UK). Tween-20. (Becton-Dickinson-PHARMINGEN. Cowley. Oxford. UK).

2.15.2 Protocol.

1. 96 well Maxisorb ELISA plates were coated with the primary antibody (anti-IgG1 clone: MC003, at a concentration of 1.25 µg/ml in carbonate buffer (100µl/well).
2. The plates were then covered with seal and left in the fridge for overnight incubation.
3. After overnight incubation the plates were washed four times with wash buffer.
4. Each well was then blocked with 200µl of wash buffer and incubated for 30 mins at room temperature.
5. Plates were then washed three times and 100µl of the standards and the B-cell supernatants were added per well.
6. The top standard concentration was 360 ng/ml while human serum was used each time as a quality control at 1/100 dilution.
7. The standards and supernatants were left to incubate for 2 h. at 37°C and then washed three more times.
8. Alkaline phosphatase labelled secondary antibody: anti-IgG1, was added at 1/500 dilution, 100µl/well, and the plates were incubated for a further 1h. at 37°C.
9. The plates were finally washed five times and 1 p-Nitrophenyl phosphate solution was added at a concentration of 1mg/ml at 100µl/well.
10. The plates were incubated in the dark for at least $^{1}/_{2}$ h. in order to allow for the yellow colour to develop.

11. The OD values and their corresponding concentration levels were measured and calculated by the ELISA Macintosh programme: SOFTMAX.

2.16 IgG2, IgG3 and IgG4 ELISAs.

The levels of these antibody isotypes were measured by the use of pre-calibrated ELISA kits. The kits, stored at 4°C , were exposed to room temperature approximately 60 min. prior to their use. The calibrator (standard), sample and control dilutions were prepared immediately before use. . In all three ELISAs the method followed was identical.

2.16.1 Reagents.

IgG2, IgG3 and IgG4 Quantitation ELISA KITS. (The Binding Site Ltd. Birmingham, UK). Buffer chemicals. (SIGMA. Pool, Dorset, UK).Human myeloma IgG1. (Calbiochem. Beeston, Nottingham, UK).Phosphate Buffered Saline (PBS). (INVITROGEN Plc. Inchinnan Business Park, Paisley, UK). Tween-20. (Becton-Dickinson-PHARMINGEN. Cowley, Oxford, UK).

2.16.2 Protocol.

1. The calibrator and control solutions were diluted 1/100 while the samples were used neat. 100 µl of each calibrator dilution, diluted control and sample were added to the appropriate wells.
2. The wells were incubated for 2h. at 37°C and then washed with wash buffer three times in order to remove all unbound proteins.
3. 100 µl of each diluted conjugate, (dilution 1/100), was added to each of the respective ELISA wells and the plates were then incubated at 37°C for 2 hours, followed by three washing cycles with the wash buffer.

4. The bound conjugates were visualised with 3,3`, 5,5` tetramethylbenzidine (TMB) substrate. The latter is added at 100 µl/well and the plates incubated for 30 min. at room temperature. TMB substrate gives a blue reaction product, the intensity of which is proportional to the concentration of IgG subclass antibody in the sample.
5. Finally, phosphoric acid is added at 100 µl/well in order to stop the reaction producing a yellow end point colour. The absorbance was measured at 450nm.

2.17 Cytokine ELISAs

The levels of IL-2, IL-6, IL-10, IL-12, IFN-γ and TGF-β were measured by direct sandwich cytokine ELISAs.

2.17.1 Reagents.

All antibody pairs, recombinant human cytokines, Tween 20. (Becton-Dickinson. PHARMINGEN. Cowley, Oxford, UK). 1 p-nitrophenylphosphatase tablets, Bovine Serum Albumin (BSA), buffer chemicals. (SIGMA. Pool, Dorset, UK). Phosphate Buffered Saline (PBS). (INVITROGEN Plc. Inchinnan Business Park, Paisley, UK).

2.17.2 Protocol.

1. Maxisorp ELISA plates were coated with 100µl/well of anti- IL-2, IL-6, IL-10, IL-12, IFN-γ and TGF-β respectively at 1µg/ml in carbonate buffer and incubated overnight at 4°C.

2 The plates were then washed 3 timed with wash buffer, and were then blocked with 100µl blocking buffer (0.5% BSA/PBS) for 30 min at 37°C.

3.Plates were washed 3 times as above and 100µl of the respective standards and samples were added.

4. After a 2h incubation at ambient temperature. plates were washed 3 times as above and 100µl/well of the corresponding biotinylated antibody were added at 1µg/ml.

5.After a further 2h incubation at room temperature and three more washes. plates were incubated with 100µl/well of streptavidin alkaline phosphatase at 1:1000 dilution for 1 h.

6.Plates were washed three more times and the substrate solution was added at 100µl/well.

7.OD values and their corresponding concentration levels were measured and calculated by the ELISA Macintosh programme: SOFTMAX™ (Version 2.35. Molecular Devices Corporation).

2.18 Optimisation of the cytokine neutralising antibodies.

The optimum concentration of the cytokine neutralising antibodies was found by titrating the latter with their relevant cytokines in B cell cultures and investigating their blocking potential on B-cell proliferation and IgE production.

2.18.1 Reagents.

Recombinant human cytokines. cytokine neutralising antibodies, Tween 20. (Becton-Dickinson-PHARMINGEN. Cowley. Oxford. UK). Phosphate Buffered Saline (PBS). (INVITROGEN Plc. Inchinnan Business Park, Paisley, UK). Alkaline phosphatase-anti-IgE polyclonal antibody, 1 p-nitrophenylphosphatase tablets, Bovine Serum Albumin (BSA), buffer chemicals. (SIGMA. Pool, Dorset. UK). Human myeloma IgE. (Calbiochem. Beeston, Nottingham, UK). Human anti-IgE monoclonal antibody (Clone: 577C1). (Gift by Dr. Darren Wheeler. AVENTIS. New Jersey. USA). AntiCD40 monoclonal antibody (Clone: 28.5). (Gift by Dr. Darren Wheeler. AVENTIS. New

Jersey, USA). AET-treated Sheep Red Blood Cells. (Described before). Fetal Calf Seum (FCS). (Harlan SERA-LAB. Belton, Loughborough, UK). (anti-IL-4: Isotype:Rat IgG2a. Clone:MQ1-17H12. Anti-TGF-β: Isotype: Mouse IgG1. Clone: 9016.2 anti-IFN-γ: Isotype:Mouse IgG1. Clone:B27. Anti-IL-6: Isotype: Rat IgG1. Clone:MQ2-13A5. Anti-IL-10: Isotype: Rat IgG2a. Clone:JES3-19F1).

2.18.2 Protocol

1. Tonsilar B cells were cultured for 10 days at 37°C under standard conditions for IgE switching: IL-4 (20 ng/ml) and anti-CD40 (0.5μg/ml) (Control).
2. At day 0, one extra cytokine (IL-6, IL-10, IFN-γ or TGF-β) was added at a concentration of 100 U/ml together with titrating amounts of its relevant cytokine neutralising specific antibody. Experiment was carried out in triplicates. The concentration of the cytokine neutralising antibodies was ranging from: 0-10,000ng/ml.
3. After a 4 day incubation, B-cell proliferation was assessed as described before.
4. After a 10 day incubation, supernatants were collected and their IgE levels were measured by an IgE sandwich ELISA.

2.19 Surface Ig Profile of purified human B cells.

The membrane bound Ig molecules on the tonsillar B cells were identified by surface two-colour staining followed by flow cytometry analysis.

2.19.1 Reagents.

AET-treated Sheep Red Blood Cells. Phosphate Buffered Saline (PBS). (INVITROGEN Plc. Inchinnan Business Park, Paisley, UK). Bovine Serum Albumin (BSA). buffer chemicals. (SIGMA. Pool, Dorset, UK). FITC-IgM, FITC-IgG, FITC-IgE, FITC-IgA, FITC-IgD, PE CD19+. (Becton-Dickinson – PHARMINGEN . Cowley, Oxford, UK). Fc-Block (Human IgG). (Miltenyi Biotec. Bisley, Surrey, UK).

2.19.2 Protocol.

1. Tonsillar B cells were purified by T-cell rosetting and added in flow cytometry tubes at $5x10^5$cells /tube and washed once with PBS-0.5%BSA for 5 min. at 200g.
2. Double surface staining was the carried out by adding 10 µl of FITC-CD19 antibody with 10 µl of a PE-conjugated Ig antibody (either IgA or IgG or IgD or IgM or IgE).
3. Together with the flow cytometry antibodies, 1 µl of Fc blocker was added per tube.
4. The tubes were incubated at 4°C for 20min and then washed once with PBS-0.5% BSA for 5 min. at 200g. and the purity was tested by flow cytometry.

2.20 IL-4R expression on B cells.

The effects of certain cytokines in the regulation of the IL-4 receptor expression on tonsillar B cells was studied as a control for the IgE synthesis process. B cells cultured with different cytokines were collected at various time points, washed and stained with biotinylated IL-4 (specific for the IL-4 receptor), followed by PE-conjugated streptavidin. The IL-4R. expression was measuerd by flow-cytometry.

2.20.1 Reagents.

Iscove's Modified Dulbecco's Medium (IMDM), L-Glutamine, human holo-transferrin, Ficoll Hypaque, NaCl powder, Penicillin/Streptomycin, alkaline phosphatase conjugated anti-IgE (SIGMA. Pool, Dorset, UK). AET-treated Sheep Red Blood Cells (previously prepared). Recombinant human cytokines, biotinylated IL-4, PE-conjugated streptavidin, FITC-CD19+ antibody (Becton-Dickinson-PHARMINGEN. Cowley, Oxford, UK). Anti-CD40 monoclonal antibody (Clone: G28.5. (Gift by Dr. Darren Wheeler. AVENTIS. New Jersey, USA). Foetal Calf Seum (FCS). (Harlan SERA-LAB. Belton. Loughborough, UK).

2.20.2 Protocol.

1. Tonsillar B cells were purified by T-cell rosetting and cultured at standard conditions (control) or with one exogenous cytokine (IL-6, IL-10, IL-2, IL-12, TGF-β or IFN-γ) at 100U/ml.
2. Supernatants from all culture conditions were collected at days 2, 4, 6 and 8 and the B cells were stained for the IL-4 receptor.
3. B-cells were added at $5x10^5$cells/tube and 500 µl of PBS with 10% FCS (pH:4.1) was added in the tubes.
4. B cells were incubated at room temperature for 3 min. and then washed twice with PBS-1%FCS. This would strip any excess IL-4 bound on the B-cell IL-4 receptors present since day 0 of the experiment.
5. 10 µl of biotinylated IL-4 was then added in each tube and the cells were incubated for 30 min. at 4°C followed by the addition of 10 µl of PE-conjugated streptavidin and FITC-conjugated CD19 antibody.
6. A further incubation for 20 min. at 4°C took place and finally the cells were washed once with PBS-1%FCS. The IL-4 receptor expression on the B cells was estimated by flow cytometry.

CHAPTER 3:PLEIOTROPIC EFFECTS OF CYTOKINES ON Ig CLASS PRODUCTION. DEVELOPMENT OF THE IgE SYSTEM.

3.1 INTRODUCTION.

B cells can express different immunoglobulin heavy chain isotypes, depending on their differentiation stage or activation state (297). B cells emerging from the bone marrow are IgM / IgD double positive and although considered as mature cells they are still immunologically "naïve" as they would not have encountered any antigen. According to the immunological status of the individual, B cells will undergo isotype switching in which new Ig molecules are secreted or expressed on the surface of the B cell. This is a T-cell dependent process and takes place in the secondary lymphoid organs such as the lymph nodes and the spleen. It is been suggested that Ig class switching is related to the number of B cell divisions, a process that is independent of division rate (196). The process of switching demands replication of the B-cell DNA that would aid the latter to go through the recombination stages of switching. Consequently, there should be a number of cell divisions before the cells switch, although this process is independent of division rate.

In the case of IgE switching the direct effect of IL-4 on the B cells is widely established (298), (299), (300), (111), (301) and (302). It has been suggested that IL-4 acts by opening up DNA switch regions for recombination rather than by regulation of isotype-specific recombinases (299). Binding of IL-4 to its receptor causes the activation of the signal transducer and activator of transcription 6 (STAT 6) and its translocation to the nucleus (303), where it binds to an IL-4-responsive element in the promoter region 5' of the Iε exon (304).

The importance of CD40-CD40L interaction between B-T cells in Ig switching is also widely established (305), (306), (307), (160), (308), (166) and (309). CD40, a member of the tumor necrosis factor receptor superfamily, has a general activating effect leading to the secretion of cytokines that will further modulate the immune response (160). Engagement by CD40L (on activated T cells), or by anti-CD40 monoclonal antibody (used in this system), provides the secondary signal leading to the survival and differentiation of B cells including isotype class switching, which is further specified by the prevailing cytokine milieu at each given time (306). Mutation of the CD40L gene results in a defective CD40-CD40L interaction, characteristic of the X-linked hyper-IgM syndrome in which B cells are rendered unable to switch to any other Ig isotype, due to the lack of CD40L expression on the T cells (307).

In this study, tonsillar B cells were isolated by either T-cell rosetting or by positive selection (CD19+ dynabeads) and their membrane Ig profile determined. The efficiency of the two purification methods was assessed on the basis of B-cell proliferation and IgE secretion. Development of the IgE system involved the titration of IL-4 and anti-CD40 in the B-cell cultures as well as the time-course of IgE secretion. Further investigation of other cytokines as potential switch factors to immunoglobulin molecules including: IgG1-4 and IgA was carried out.

Anti-CD40 antibody was always present in the study of Ig isotype secretion as it`s a prerequisite for B-cell survival and differentiation. Cytokines that were found to have an effect on IgG1-4 and IgA subclass switching were tested in the absence of IL-4 in order to distinguish between clonal expansion of precommitted B cells and actual stimulation of Ig production. The presence of IL-4 has a proliferative effect on B cells (310) and (311), hence it can be involved in their clonal expansion (185). Cytokines that enhanced

the secretion of Ig isotypes in the absence of IL-4, were considered to have a direct effect on the human B cells, as their individual proliferative activity on the latter is minimal, if any, compared to IL-4.

3.2 RESULTS

3.2.1 Purification of tonsillar B cells.

B cells were purified from a tonsillar cell suspension by either positive selection (CD19+ dynabeads) or T-cell rosetting (Sheep Red Blood Cells). Following purification the levels of B-cell purity were assessed by flow cytometry. B cells were stained by anti-CD19-PE conjugated monoclonal antibody. T cell impurities were identified by CD3-FITC conjugated antibody, while the monocyte impurities were determined with anti-CD14-FITC conjugated antibody. B-cell purity levels were similar between the two purification techniques, since by using anti-CD19 dynabeads purity was around 99%, while T-cell rosetting gave purity levels of around 96% (Figure 3.1 and Table 3.1).

3.2.2 Membrane Ig (mIg) profile of tonsillar B cells.

Tonsillar B cells isolated by T-cell rosetting were surface stained with anti-CD19-PE antibody and one from each FITC-conjugated Ig antibodies from: IgM, IgD, IgG, IgA and IgE. Flow cytometry analysis showed that 56% of the total B cell population was IgM+, 18% IgD+, 11% IgG+, 27% IgA+ and only 5% IgE+ (Table 3.2). The total B cells stained was 99% which indicates that all IgD+ B cells were probably also IgM+.

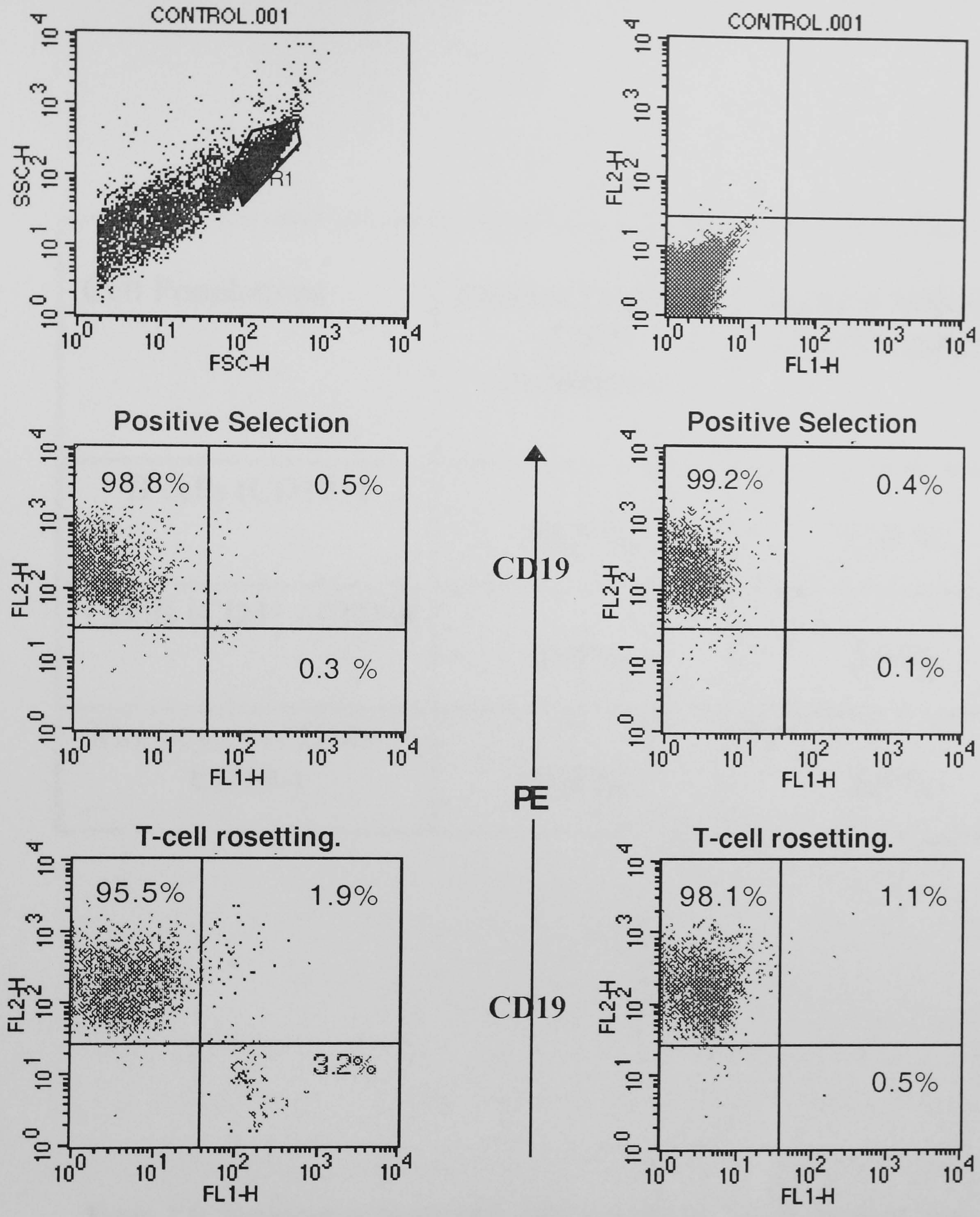


Fig.3.1: **B-CELL PURITY.** Tonsillar B cells were isolated by either T-cell rosetting or by positive selection using CD19+ dynabeads. The B-cell purity levels were measured by cell surface monoclonal antibodies. Anti-CD19 PE was used for B-cell staining, anti-CD3 FITC for T-cell staining and anti-CD14 FITC for monocyte staining. The analysis showed similar levels of B-cell purity obtained by the two methods.

Cell Populations	Positive Selection (CD19 dynabeads)	Negative Selection (T-cell rosetting)
B cells (CD19+)	98.8 %	97.9 %
T cells (CD3+ / CD19-)	0.8 %	2.2 %
Monocytes (CD14+ / CD19-)	0.5 %	0.5 %

Table 3.1: Purification levels of B cells isolated by T-cell rosetting (negative selection) or CD19+ dynabeads (positive selection). Tonsillar B cells were isolated by the two purification techniques and the levels of B cells, T cells and monocytes were measured by the relevant cell surface monoclonal antibodies. The table presents the mean values of the cell populations from three different experiments.

% mIgM+	% mIgD+	% mIgG+	% mIgA+	% mIgE+
56	18	11	27	5

TOTAL B CELLS STAINED = 99%

Table 3.2: Membrane Ig (mIg) profile of freshly isolated tonsillar B cells. Tonsillar B cells were isolated by T-cell rosetting and surface stained with flow cytometry antibodies. Double staining was carried out with FITC anti-CD19 monoclonal antibody and PE-conjugated isotype-specific monoclonal antibodies (i.e.: anti-IgG, anti-IgM, anti-IgE, anti-IgD and anti-IgA). The cells were incubated also with a human Fc-blocker in order to minimise any non-specific binding. The mIg levels on the tonsillar B cells were measured by flow cytometry.

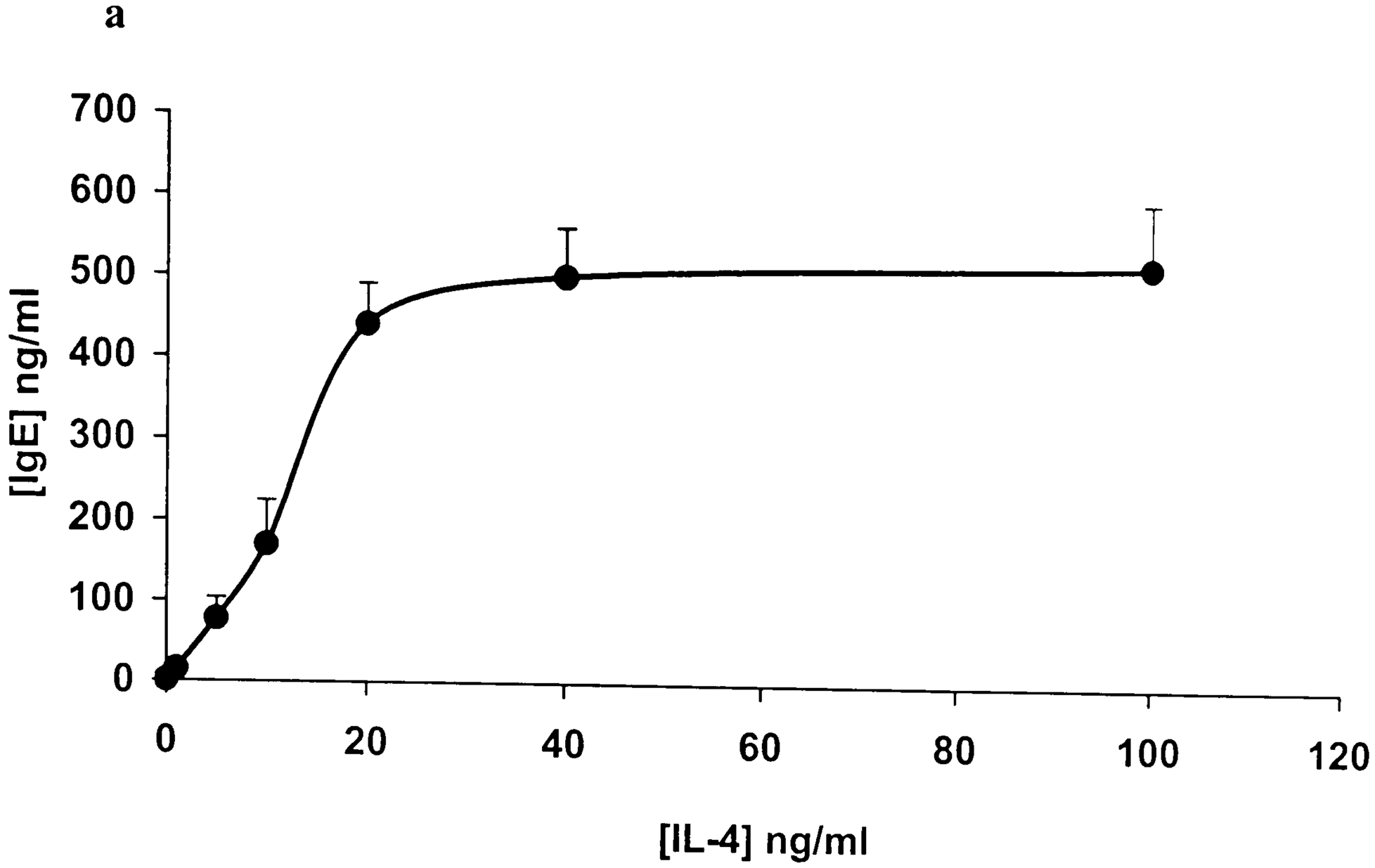
3.2.3 Optimisation of the IgE system by human B cells in vitro

Purified B cells were cultured for 10 days with different concentrations of IL-4 and anti-CD40. IgE was measured in the culture supernatants after 10 days. From these results it was concluded that the optimum IL-4 concentration for significant IgE production was 20 ng/ml (IgE: 450 ng/ml) while that of anti-CD40 was found to be 0.5µg/ml (IgE: 350 ng/ml) (Figures 3.2.1 and 3.2.2). No IgE was detected in the absence of IL-4 and anti-CD40, while higher amounts of 20 ng/ml of IL-4 and 0.5 µg/ml of anti-CD40 had negligible effects on the IgE levels secreted by the B cells.

These conditions were used in all subsequent experiments (unless stated otherwise) and became the core of the IgE class switching system used.

3.2.4 Time course of IgE secretion by human B cells in vitro

Tonsillar B cells were cultured with IL-4 (20 ng/ml) and anti-CD40 (0.5 µg/ml) and the supernatants were collected at days: 1, 4, 8, 10, 14 and 18. The levels of IgE in the supernatants were measured by an IgE ELISA. IgE levels increased with time most rapidly between days 8 – 10 (600 – 1300 ng/ml) and reached a plateau by day 10 (Figure 3.3). Prior to day 2, no IgE could be detected in the B-cell cultures.



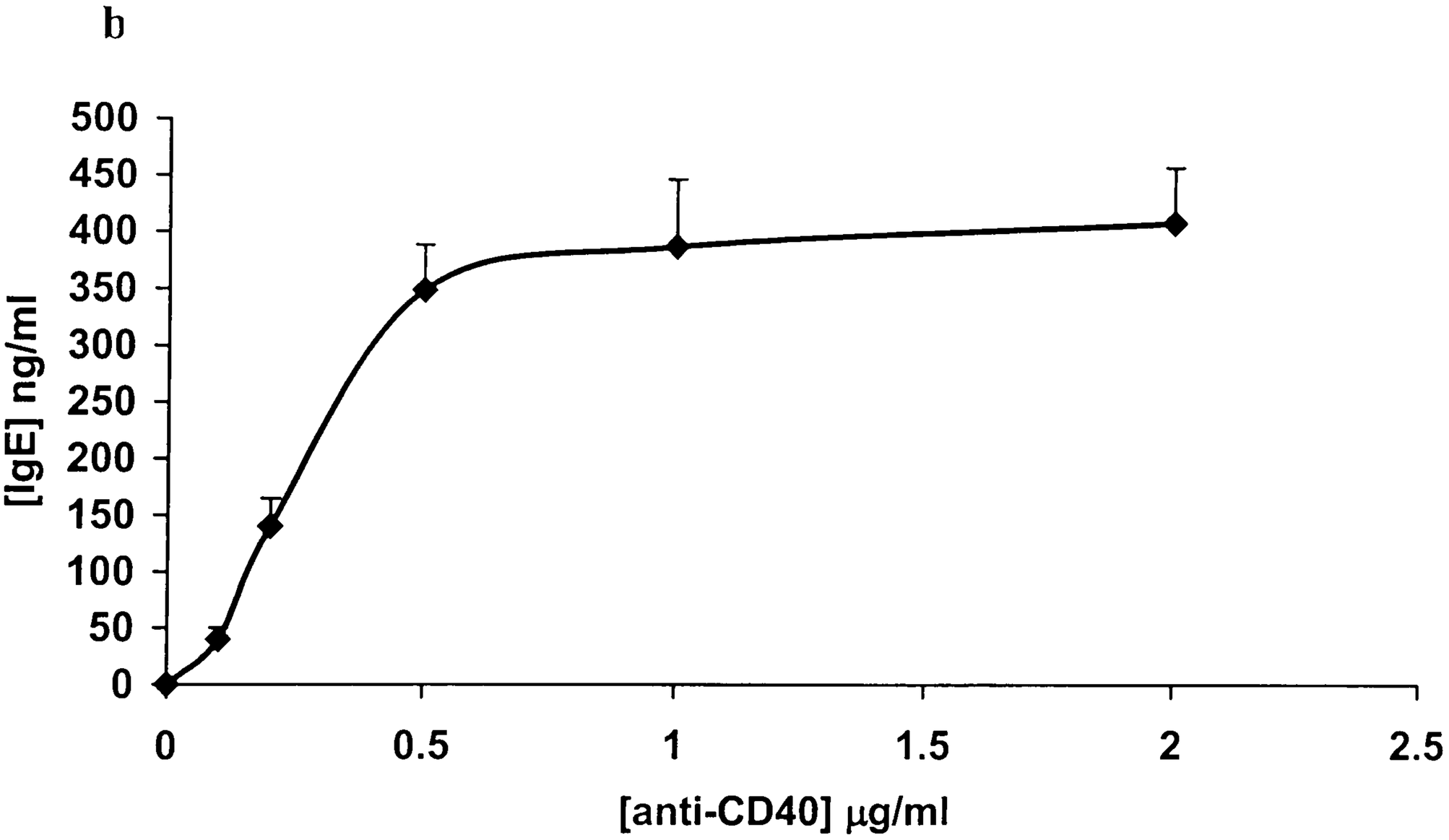


Fig.3.2a: Optimisation of IL-4 concentration in the IgE system. Tonsillar B cells were isolated by T cell rosetting and cultured at a concentration of 10^5 cells/well, with increasing amounts of IL-4 and a constant concentration of anti-CD40 (0.5µg/ml). After 10 days, supernatants were collected and the IgE levels measured by an IgE sandwich ELISA and are expressed as the mean of three donors ± SEM IgE.

Fig.3.2b: Optimisation of the anti-CD40 concentration in the IgE system. Tonsillar B cells were isolated by T cell rosetting and cultured at a concentration of 10^5 cells/well, with increasing amounts of anti-CD40 and a constant concentration of IL-4 (20 ng/ml). After 10 days, supernatants were collected and the IgE levels measured by an IgE sandwich ELISA and are expressed as the mean of three donors ± SEM IgE.

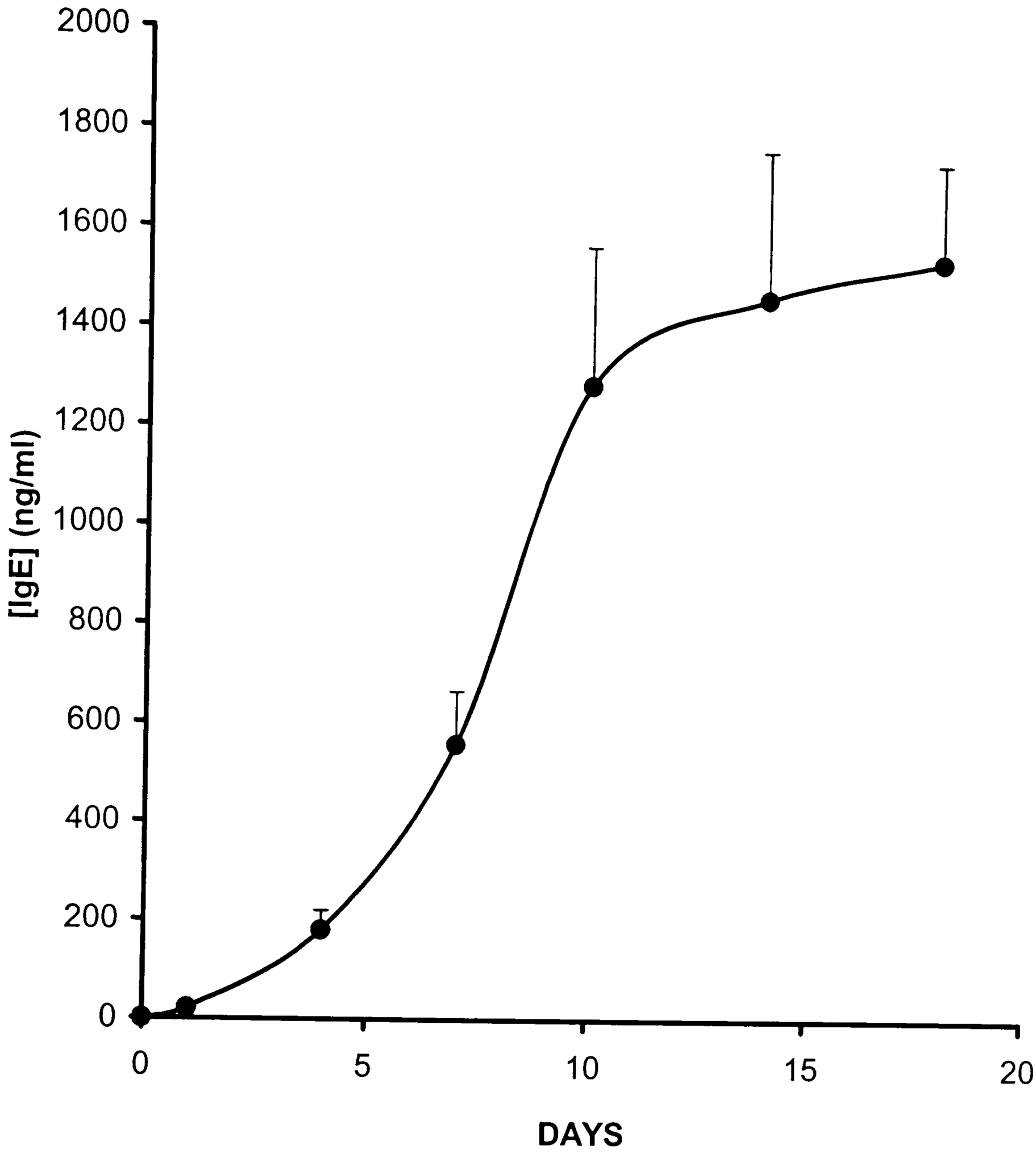


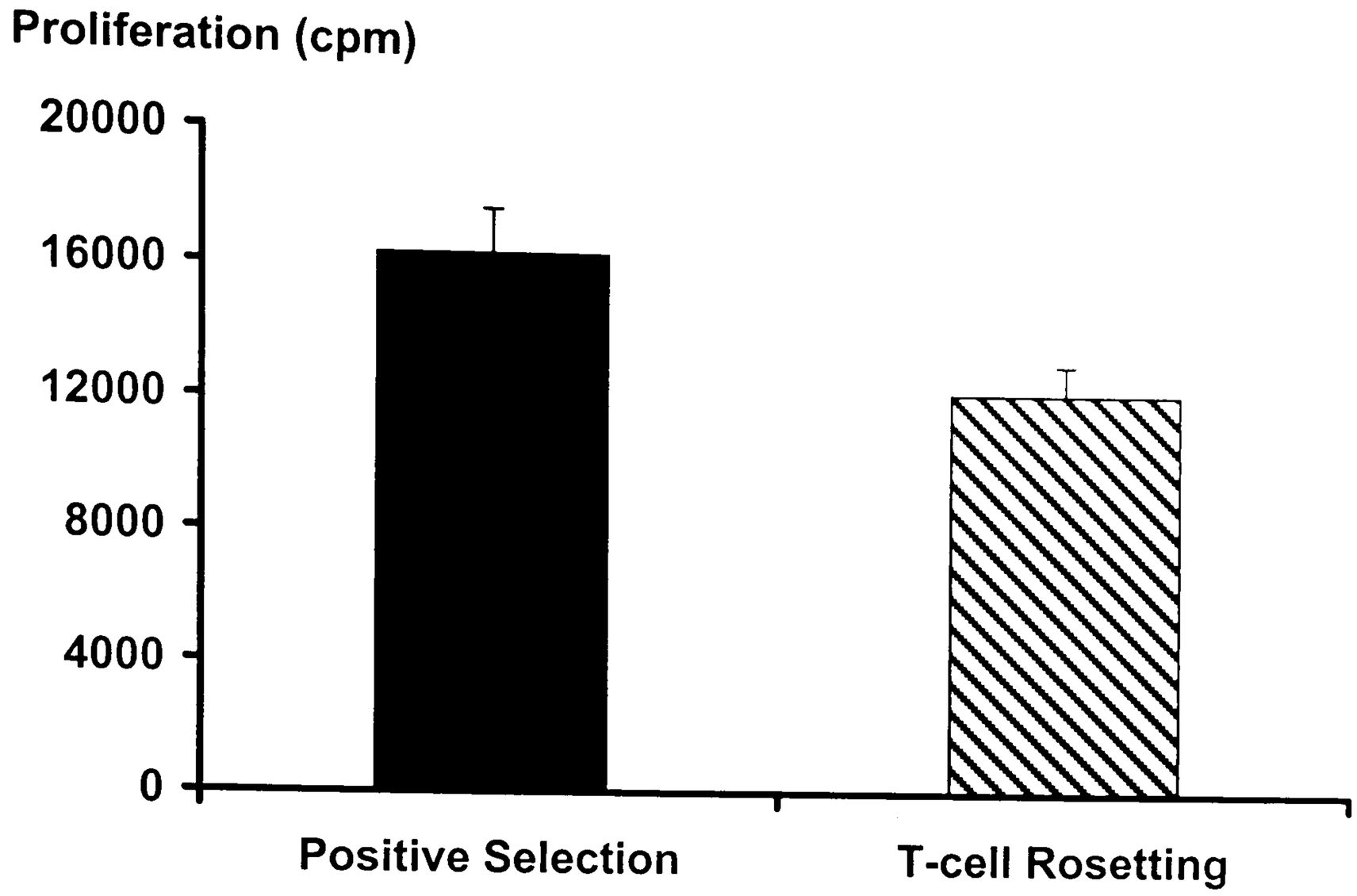
Fig.3.3: Time course of IgE secretion by tonsillar B cells "in vitro". Tonsillar B cells were purified by T-cell rosetting and cultured at a concentration of 10^5 cells/well, with IL-4 (20ng/ml), anti-CD40 (0.5μg/ml). The supernatants were collected at days: 1, 4, 8, 10, 14 and 18 and the levels of IgE were measured by an IgE sandwich ELISA. The IgE levels are expressed as the mean of three donors ± SEM. The IgE levels in the supernatants increased with time, reaching a plateau after day 10

3.2.5 Effect of purification method on B-cell proliferation and IgE secretion.

Tonsillar B cells were purified by positive selection (CD19+ dynabeads) and T-cell rosetting. Both sets of purified cells were cultured with IL-4 (20 ng/ml) and anti-CD40 (0.5 μg/ml). At day 4, plates were pulsed with ^{3}H-Thymidine in order to measure B-cell proliferation. At day 10, supernatants were collected and the IgE levels secreted were measured by a direct ELISA. B cells purified by T-cell rosetting secreted higher levels of IgE compared to B cells that were positively selected with the CD19+ dynabeads. The opposite was observed with the levels of B-cell proliferation. (Fig. 3.4)

3.2.6 Purification of CD4+ / CD8+ tonsillar T cells.

CD4+ and CD8+ T cells were also isolated by positive selection using CD4+ and CD8+ dynabeads respectively. The purity levels of the CD4+ and CD8+ T cells was assessed by flow cytometry. Both T-cell populations were stained with CD4-PE conjugated antibody as well as CD8-FITC conjugated antibody. 97.8% of the CD4+ T cells were CD4+/CD8- while 93.2% of the CD8+ T cells were CD8+/CD4- (figure 3.5).



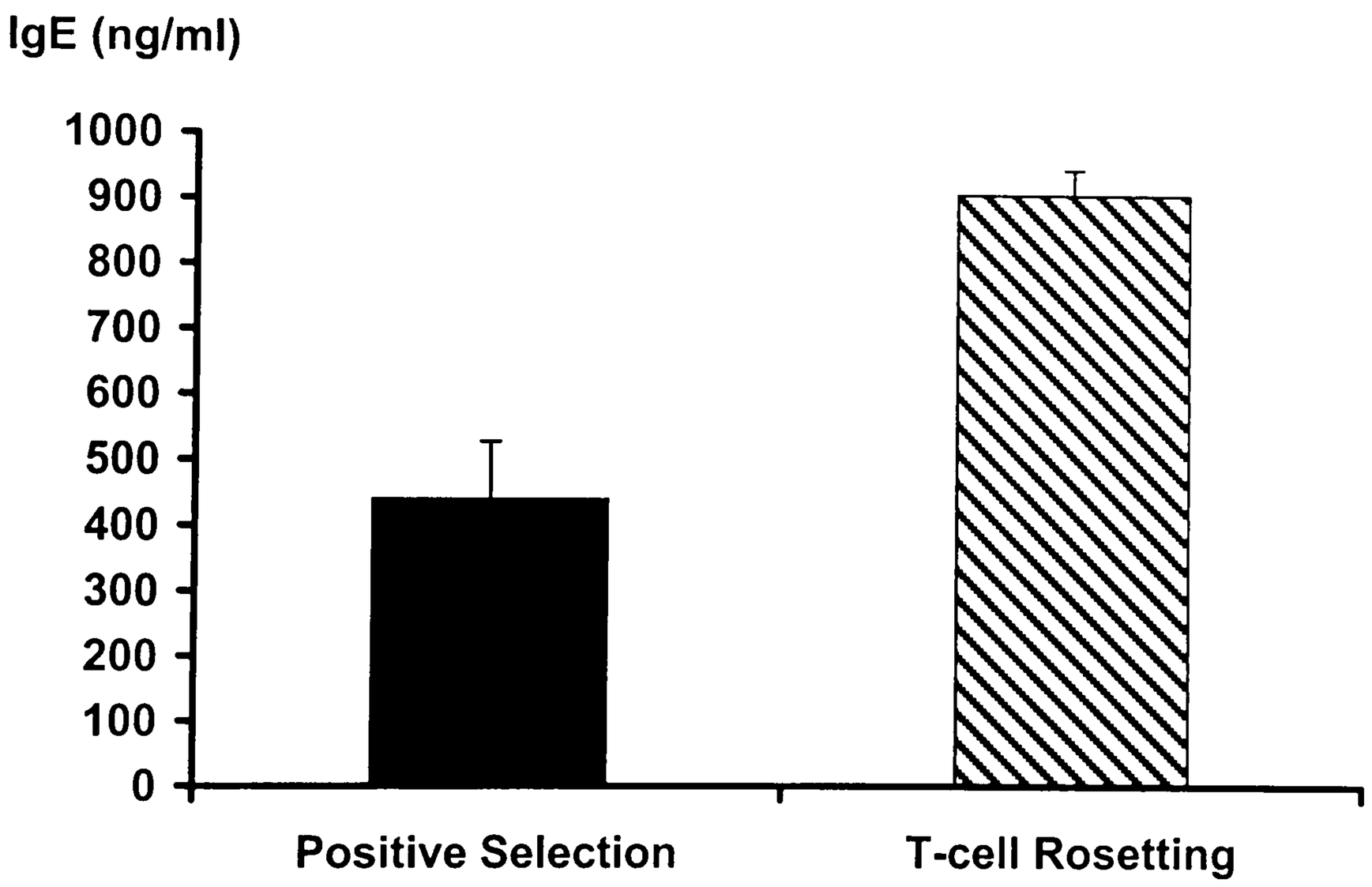


Fig. 3.4: Effect of the purification method on B-cell proliferation and IgE secretion "in vitro". Tonsillar B cells were purified by positive selection (CD19+ dynabeads) or by T-cell rosetting and cultured with IL-4 (20 ng/ml) and anti-CD40 (0.5 μg/ml). At day 4, culture plates were pulsed with ^{3}H-Thymidine and B-cell proliferation was assesed. At day 10, supernatants were collected and the levels of IgE were measured by ELISA. The results shown are the average of three experiments and the standard errors have been calculated.

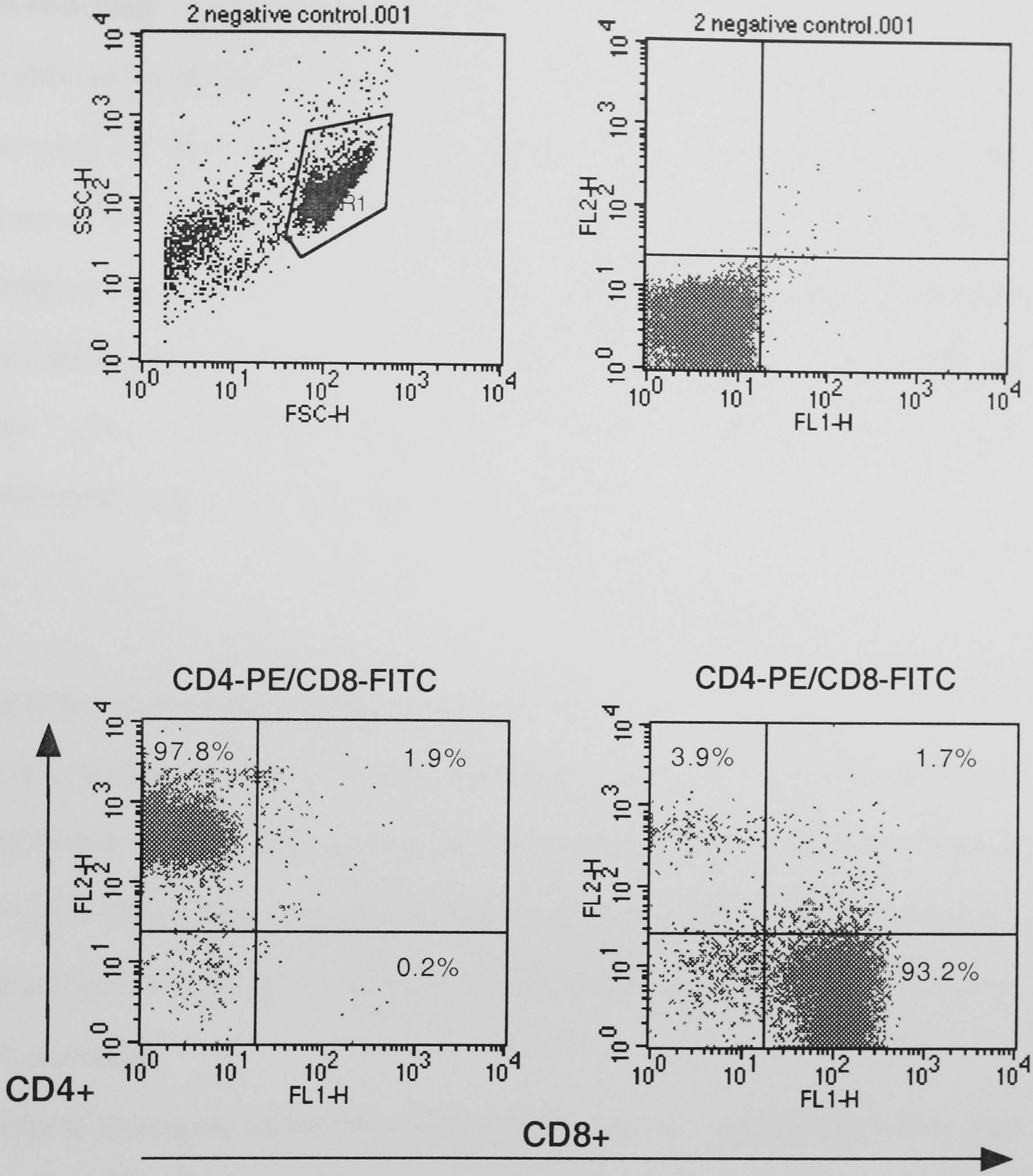


Fig. 3.5: Tonsillar CD4+ / CD8+ T-cell purity. CD4+ and CD8+ T cells were positivelly selected from a tonsillar cell suspension using anti-CD4+ and anti-CD8+ dynabeads respectivelly. The purity of the CD4+ and CD8+ T cells was assessed by flow-cytometry using anti-CD4 and anti-CD8 FITC conjugated antibodies. These results are representative of three experiments.

3.2.7 Effect of autologous CD4+ and CD8+ T cells on B-cell proliferation and IgE class switching.

The effect of T-cell impurities on the B cell cultures, proliferation and IgE secretion was determined. For this purpose, B cells were cultured with IL-4 (20 ng/ml) and anti-CD40 (0.5 μg/ml) and increasing numbers of either CD4+ or CD8+ T cells were added. B-cell proliferation was assessed by the incorporation of ^{3}H-Thymidine, while the IgE levels were measured by a direct sandwich IgE ELISA. Figure 3.6 shows that both CD4+ and CD8+ T cells, up to a contamination of 10%, did not have a significant effect on B cell proliferation or IgE secretion in the cultures.

3.2.8 Effect of cytokines on IgG1 class production.

Tonsillar B cells, purified by T-cell rosetting were cultured with IL-4 (20 ng/ml) and anti-CD40 (0.5 μg/ml) (control conditions) as well as with one exogenous cytokine at 100 U/ml. After 10 days supernatants were collected and IgG1 levels measured by ELISA (Figure 3.7). IL-10, IL-12 and even more IFN-γ seemed to stimulate B cells to IgG1 production.

In order to investigate whether the effect of these cytokines was IL-4-dependent, they were titrated in the B cell cultures, in the absence of IL-4. Only IFN-γ was able to stimulate B cells to IgG1 secretion independent of IL-4. The effect of IFN-γ on IgG1 was dose-dependent and did not plateau by 1000U/ml of the cytokine. IL-10 and IL-12 had no significant effect on IgG1 secretion in the absence of IL-4 (figure 3.7).

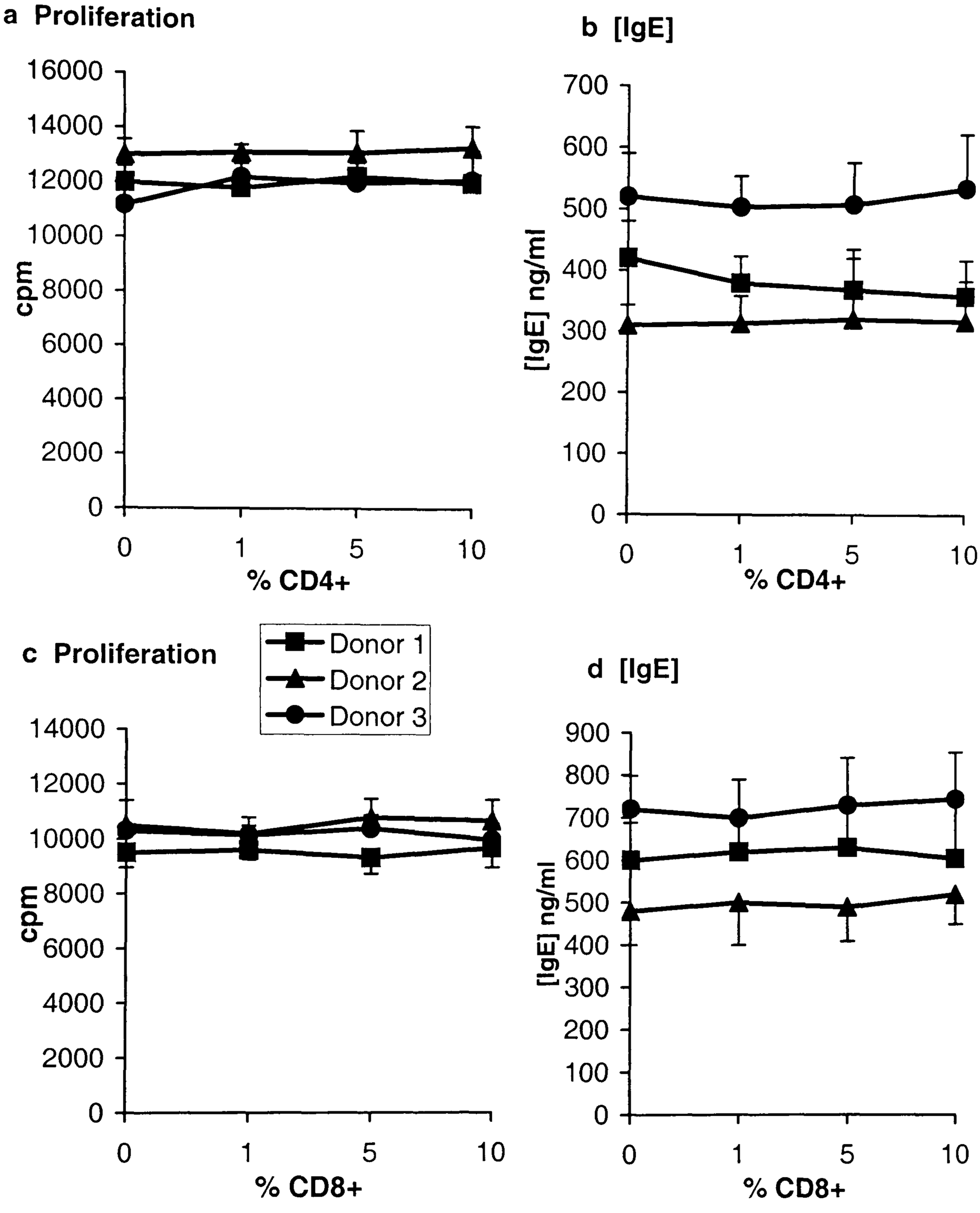


Fig. 3.6: Effect of autologous CD4+ and CD8+ T cells on the proliferation and IgE secretion by tonsillar B cells. Tonsillar B cells, CD4 and CD8 T cells were purified by positive selection using CD4 and CD8 dynabeads respectively. Two different culture plates were set up; in the first one, the CD4 T cells were titrated with the B cells, while in the second one CD8 T cells were titrated with the B cells. IL-4 (20 ng/ml) and anti-CD40 (0.5 μg/ml) were always present in the cultures. After 4 days half of the supernatants were collected and the proliferation was measured (a + c). After 10 day culture the rest of the supernatants were collected and the levels of IgE were measured by an IgE ELISA (b + d). In both cases the mean of triplicate is expressed. ±S.D.

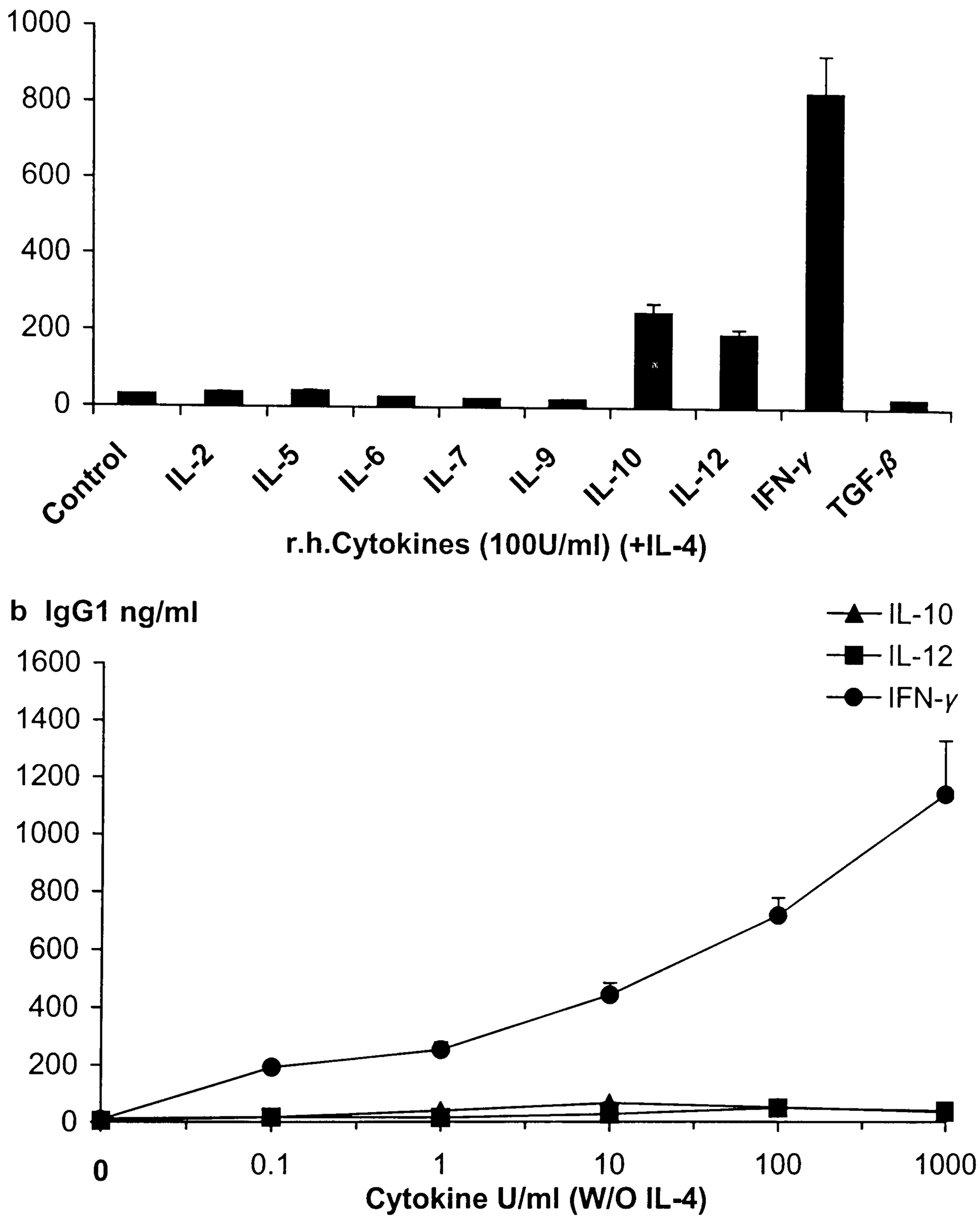


Fig.3.7: Effect of cytokines on IgG1 production by human B cells.
Tonsillar B cells were isolated by T-cell rosetting and cultured in the presence of IL-4 (20 ng/ml), anti-CD40 (0.5 μg/ml) and one exogenous cytokine at 100U/ml. After 10 days supernatants were collected and the IgG1 levels were measured by ELISA, and expressed as the mean of three donors ± SEM IgG1 (a).
Human B cells were cultured with increasing amounts of cytokines found to have an effect on IgG1 production in the absence of IL-4. After 10 days supernatants were collected and the IgG1 levels were measured by ELISA and expressed as the means of three donors ± SEM IgG1 (b).

3.2.9 Effect of cytokines on IgG2 class production.

Tonsillar B cells, purified by T-cell rosetting were cultured with IL-4 (20 ng/ml) and anti-CD40 (0.5 μg/ml) (control conditions) as well as with one exogenous cytokine at the physiologic concentration of 100 U/ml. After 10 days of culture, the supernatants were collected and the IgG2 levels were measured by ELISA. None of the cytokines tested were able to stimulate human B cells to secrete IgG2 (figure 3.8).

3.2.10 Effect of cytokines on IgG3 class production.

Tonsillar B cells, purified by T-cell rosetting were cultured with IL-4 (20 ng/ml) and anti-CD40 (0.5 μg/ml) (control conditions) as well as with one exogenous cytokine at 100 U/ml. After 10 days of culture, the supernatants were collected and the IgG3 levels were measured by ELISA. IL-10 stimulated B cells to IgG3 production only in the presence of IL-4 which indicates synergy between the two cytokines.

In order to investigate whether the effect of IL-10 was IL-4-dependent, the latter was titrated in the B cell cultures, in the absence of IL-4. IL-10 alone was unable to stimulate B cells to secrete significant amounts of IgG3 (figure 3.9).

3.2.11 Effect of cytokines on IgG4 class switching.

Tonsillar B cells, purified by T-cell rosetting were cultured with IL-4 (20 ng/ml) and anti-CD40 (0.5 μg/ml) (control conditions) as well as with one exogenous cytokine at 100 U/ml. After 10 days of culture, the supernatants were collected and the IgG4 levels were measured by ELISA. IL-6 and IL-10 stimulated B cells to secrete IgG4. IL-12.

TGF-β and IFN-γ inhibited the IL-4-mediated IgG4 secretion by the human B cells, while IL-2, IL-5, IL-7 and IL-9 had no effect.

In order to investigate whether the effect of IL-6 and IL-10 on IgG4 secretion was IL-4-dependent, the cytokines were titrated in the B cell cultures, in the absence of IL-4. Only IL-10 was able to stimulate B cells to secrete IgG4. Induction of the latter immunoglobulin by IL-10 was dose-dependent while independent of IL-4. The IgG4 stimulatory activity of IL-6 was found to be IL-4-dependent (figure 3.10).

3.2.12 Effect of cytokines on IgA class switching.

Tonsillar B cells, purified by T-cell rosetting were cultured with IL-4 (20 ng/ml) and anti-CD40 (0.5 μg/ml) (control conditions) as well as with one exogenous cytokine at 100 U/ml. After 10 days of culture, the supernatants were collected and the IgA levels were measured by ELISA. IL-10 and TGF-β stimulated B cells to produce IgA.

In order to investigate whether the effect of these cytokines was IL-4-dependent, they were titrated in the B cell cultures, in the absence of IL-4. Only TGF-β was able to stimulate B cells to secrete IgA. The effect of TGF-β was dose-dependent while independent of IL-4. IL-10 had a much lesser effect on IgA secretion in the absence of IL-4 (30ng/ml) compared to when IL-4 was present (445 ng/ml) (figure 3.11).

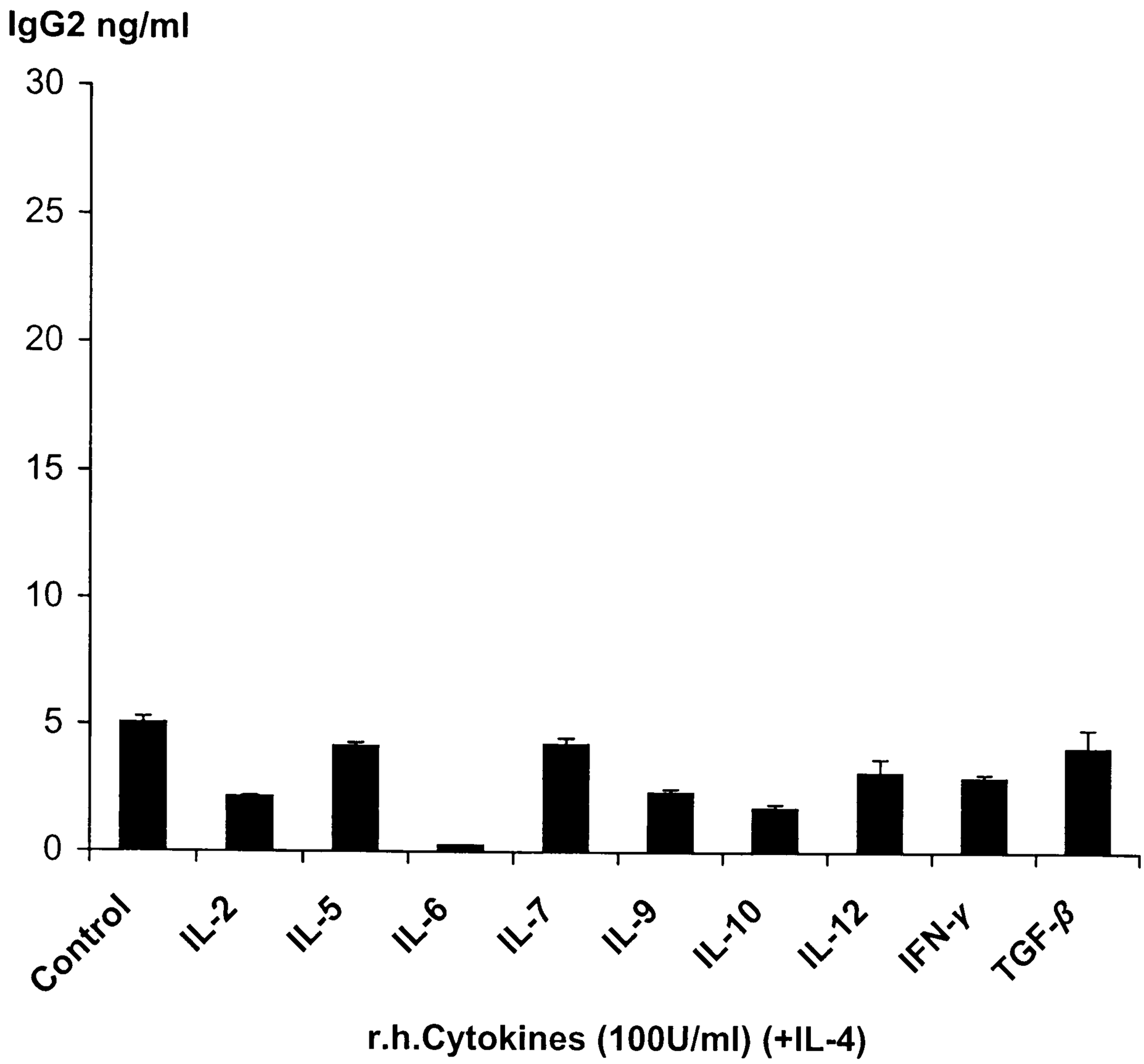
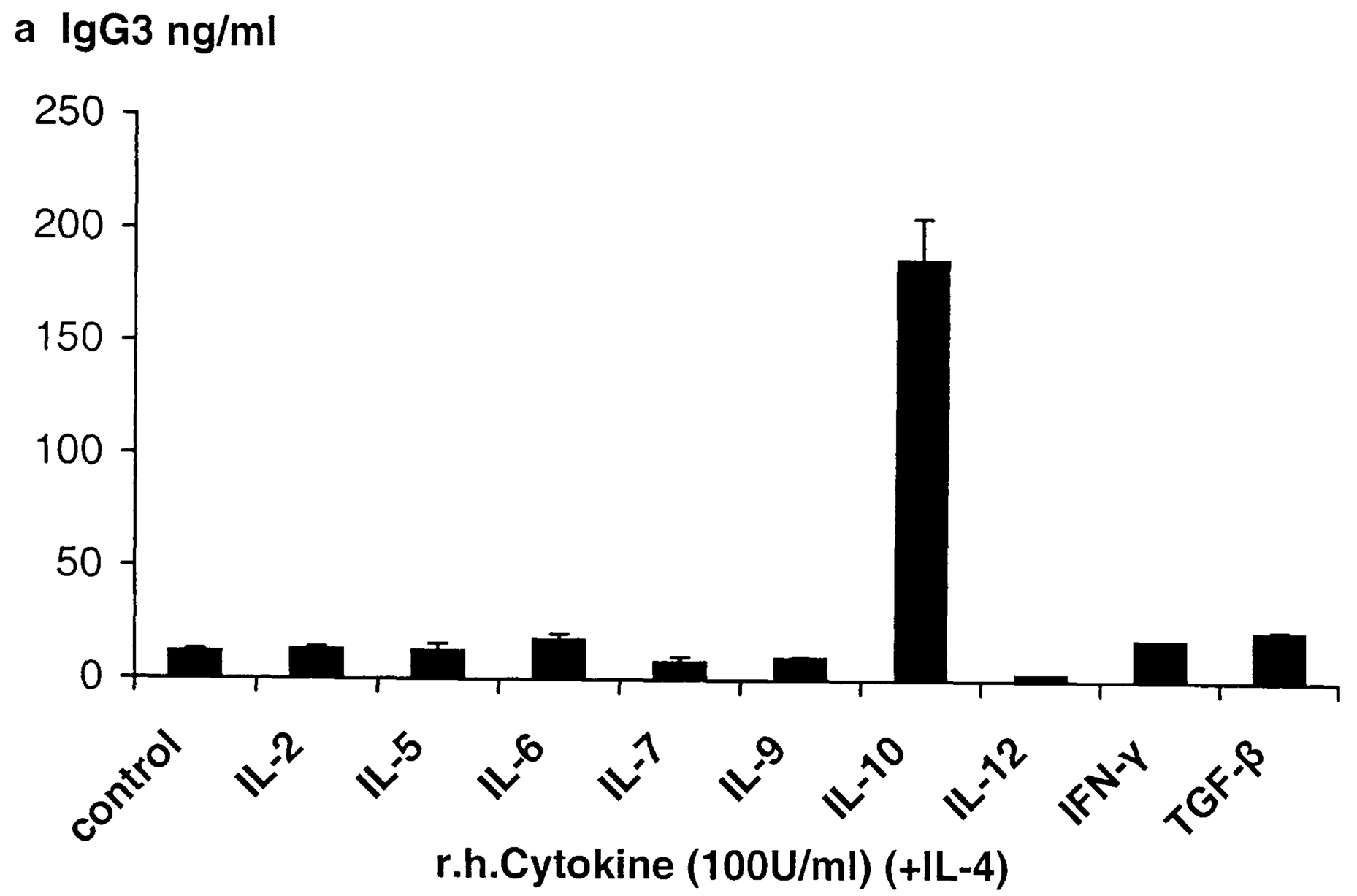


Fig.3.8: Effect of cytokines on IgG2 production by human B cells. Tonsillar B cells were isolated by T-cell rosetting and cultured in the presence of IL-4 (20 ng/ml), anti-CD40 (0.5 μg/ml) and one exogenous cytokine at 100U/ml. After 10 days supernatants were collected and the levels of IgG2 in the culture were measured by ELISA and expressed as the mean of triplicates ± S.D.



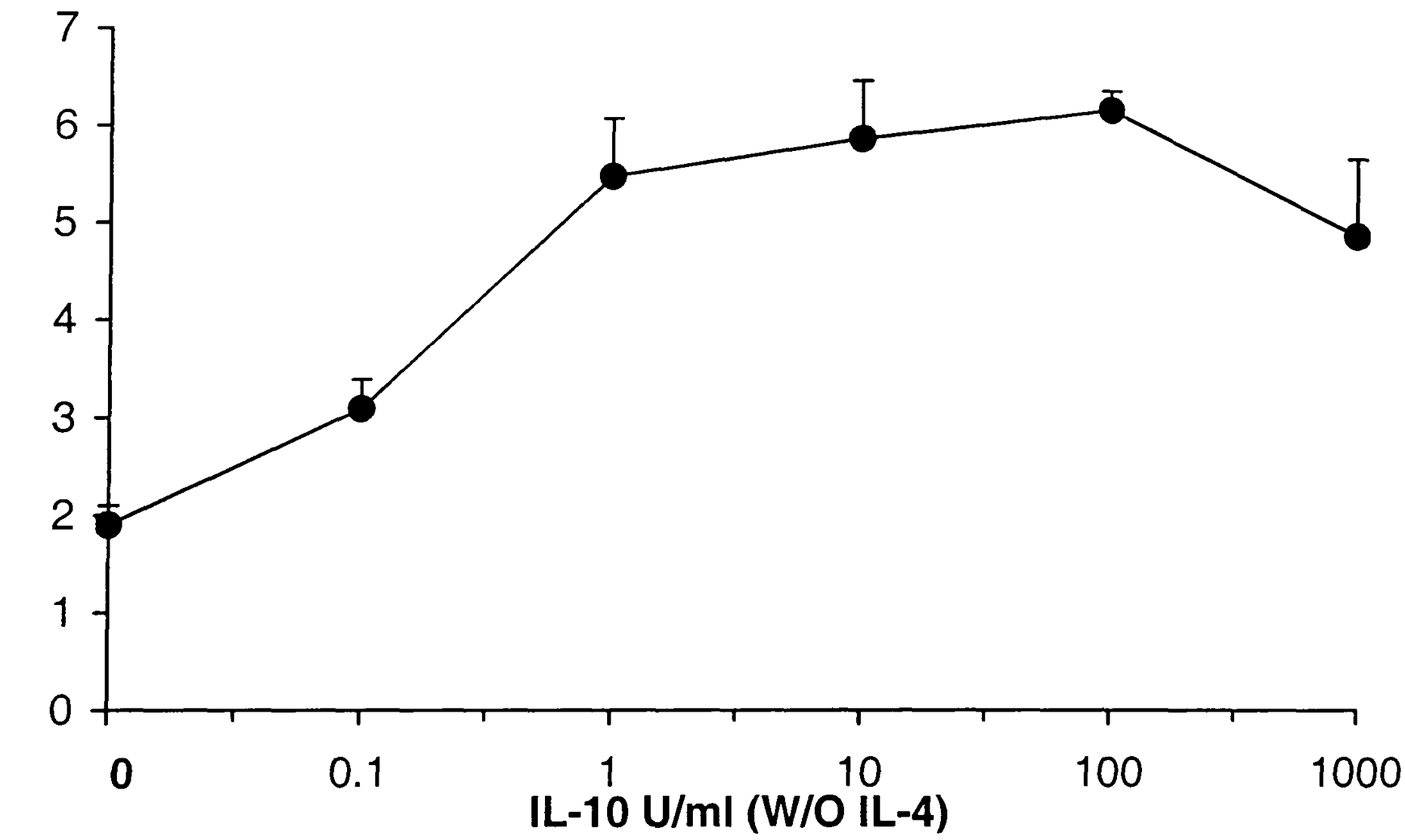


Fig.3.9: Effect of cytokines on IgG3 production by human B cells.
Tonsillar B cells were isolated by T-cell rosetting and cultured in the presence of IL-4 (20 ng/ml), anti-CD40 (0.5 μg/ml) and one exogenous cytokine at 100U/ml. After 10 days supernatants were collected and the levels of IgG3 were measured by ELISA and expressed as the mean of three donors ± SEM IgG3 (a).
Cytokines found to have an effect on IgG3 production (IL-10, in this case), were titrated in the B cell cultures in the absence of IL-4. After 10 days supernatants were collected and the IgG3 levels were measured by ELISA and expressed as the mean of three donors ± SEM IgG3 (b).

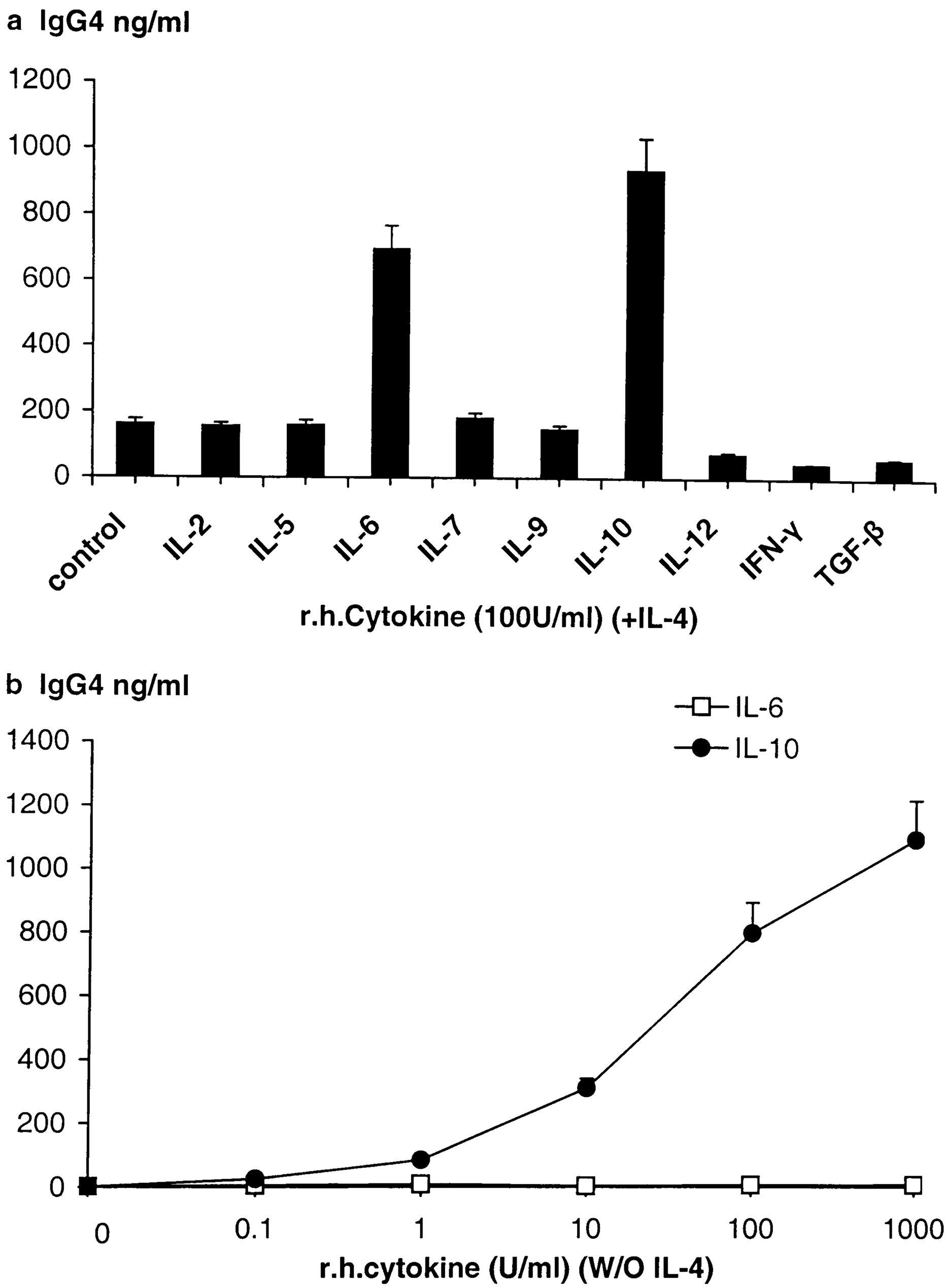


Fig.3.10: Effect of cytokines on IgG4 production by human B cells.
Tonsillar B cells were isolated by T-cell rosetting and cultured in the presence of IL-4 (20 ng/ml), anti-CD40 (0.5 μg/ml) and one exogenous cytokine at 100U/ml. After 10 days supernatants were collected and the levels of IgG4 were measured by ELISA and expressed as the mean of three donors ± SEM IgG4 (a).
Human B cells were cultured with increasing amounts of cytokines found to have an effect on IgG4 production, in the absence of IL-4. After 10 days supernatants were collected and the IgG4 levels were measured by ELISA and expressed as the mean of three donors ± SEM IgG4 (b).

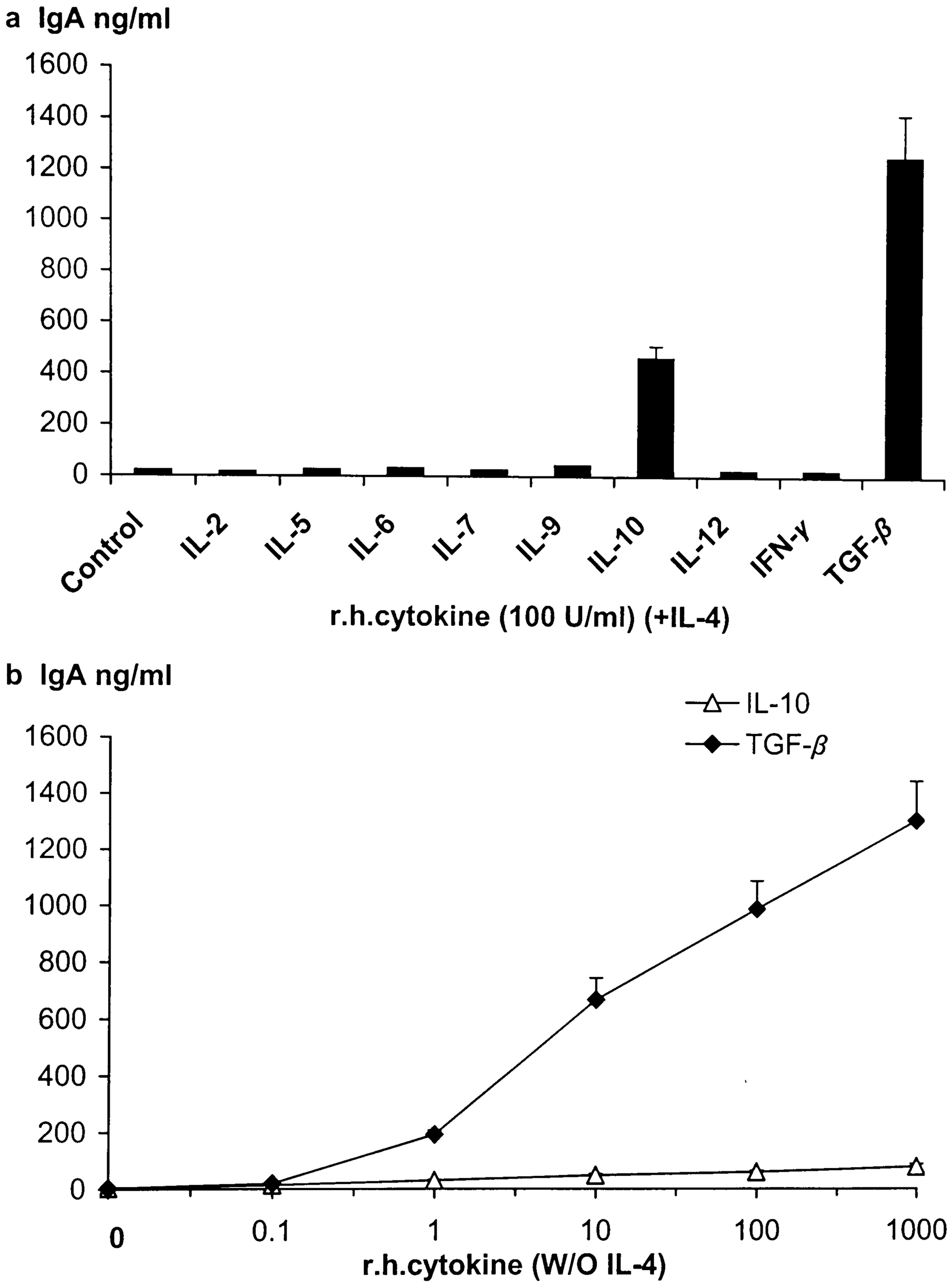


Fig.3.11: Effect of cytokines on IgA production by human B cells.

Tonsillar B cells were isolated by T-cell rosetting and cultured in the presence of IL-4 (20 ng/ml), anti-CD40 (0.5 μg/ml) and one exogenous cytokine at 100U/ml. After 10 days supernatants were collected and the levels of IgA were measured by ELISA and expressed as the mean of three donors ± SEM IgA (a).

Human B cells were cultured with increasing amounts of cytokines found to have an effect on IgA production, in the absence of IL-4. After 10 days supernatants were collected and the IgA levels were measured by ELISA and expressed as the mean of three donors ± SEM IgA (b).

3.3 DISCUSSION.

In this chapter we established the IgE system that will be used in the subsequent experiments to study IgE class synthesis. Two B cell purification methods were carried out and compared: positive selection and T-cell rosetting. The differences in the B-cell purity levels obtained with these two methods, were mainly due to T-cell impurities present in the cultures, which in the case of T-cell rosetting could reach a level of 4.5% (CD3+ cells), although the average was around 2.2%. Monocyte impurities never exceeded the levels of 2.5% (CD14+ cells). CD3+ B cells are not known to exist, thus the levels of CD3+ / CD19+ double positive cells picked up during flow cytometry analysis was considered an artefact. CD4+ and CD8+ T cells were added to the B-cell cultures, (up to 10%), but had no effect on the B-cell proliferation or IgE secretion. Despite the similar efficiency of the two purification methods, T-cell rosetting was preferred as more IgE was secreted (see figure 3.4).

Titration of IL-4 showed optimum levels according to IgE production at 20 ng/ml while the efficiency of anti-CD40 in IgE switching peaked at a concentration of 0.5 μg/ml. IL-4 can directly switch B cells to IgE production by activating specific transcription factors (e.g.:STAT 6), followed by transcription of the Iε region (312). Anti-CD40 provides the primary signal which will aid heavy-chain DNA switch recombination which in turn will position the Cε gene directly downstream of the VDJ genes making it accessible to the transcription factors (312). T-cells, or T-cell membranes can be used instead of anti-CD40, as they possess CD40L, sufficient to provide the required

secondary signal to the B cells. Recombinant CD40L in membrane vesicles has been shown to be equally effective (308).

The time-course of IgE secretion showed that at least 90% of the potential IgE secreted in the B cell cultures. is present by day 10. From this result it was decided that a 10 day culture period would give sufficient IgE levels for our study. Switching was believed to occur after at least 4 days of culture and any IgE picked up prior to that time point was attributed to the presence of precommitted B cells. This was confirmed by the presence of a small population of B cells (5%) that possessed membrane IgE (mIgE) on their surface.

In the Ig isotype secretion experiments B cells were cultured with one exogenous cytokine in the presence of IL-4. Cytokines that had an effect on the production of a specific Ig isotype were titrated to the B-cell cultures in the absence of IL-4 as the latter can lead to clonal expansion of precommitted cells. If the response was not altered, then the effect would be attributable to the action of the specific cytokine acting directly on the B cells. Where the effect was IL-4-dependent it was unclear whether clonal expansion was involved or a switching mechanism driven by the combination of IL-4 with the particular cytokine. This problem might be resolved by studying the effect of the particular cytokines on naïve B cells (IgM+/IgD+ cells), which haven't been committed to any Ig isotype. Concerning IgG1 production, IL-10, IL-12 and IFN-γ could upregulate it although only the latter cytokine was IL-4-independent. The IgG1 switching properties of IFN-γ were demonstrated early by Nakagawa and colleagues, where the addition of IFN-γ with Staphylococcus Aureus (SAC)-activated B cells lead to the production of IgG1 in the cultures. On the other hand, Briere *et al.*, (1994), showed

that IL-10 could switch CD40-activated human B cells to produce IgG1 in the absence of IL-4, a finding not in agreement with our results in which IL-10 was unable to sustain significant levels of IgG1 in an IL-4-independent fashion (313). In the case of IgG2, no cytokines were able to upregulate the secretion of this antibody. The low levels detected where either due to background readings or due to an IL-4-dependent clonal expansion of a small precommitted B-cell subpopulation. Interestingly, Calvert *et al.*, (1990), have shown that IL-2 was able to promote low levels of IgG2 secretion by LPS-stimulated tonsillar B cells (314). Kawano and Noma (1996), demonstrated synergy between IL-2 and IFN-γ for IgG2 switching by purified human B cells (315). This might be true although in our hands, these cytokines were unable to induce IgG2 production by the purified B cells individually or together with IL-4. IL-10 was capable of promoting IgG3 secretion but in an IL-4-dependent fashion. It has been also demonstrated that IL-10 could switch CD40-activated naïve tonsillar B cells to IgG3. Thus, in addition to its growth promoting and differentiating activities on human B cells, IL-10 may represent a switch factor for IgG1 and IgG3 (313).

In the case of IgG4, IL-4 was thought to induce its production. This was supported by Spiegelberg *et al.*, (1990) using B cells purified from peripheral blood and activated by anti-CD40 (316). Upregulation of IgG4 secretion due to IL-10 was dose-dependent and IL-4-independent. The possible role of IL-10 as a switch factor for IgG4 has not been suggested before. Only Jeannin et al., (1998), demonstrated that addition of IL-10 in human B cell cultures at day 0 could induce IgG4 secretion but only in the presence of IL-4 (317). Hence this increase in IgG4 production was accounted to the immunoproliferative activity of IL-10 on B cells that were switched to IgG4 synthesis by

IL-4. Interestingly, IL-12, IFN-γ and TGF-β downregulated the IL-4-dependent IgG4 secretion in the B cell cultures.

Finally, IL-10 and TGF-β were able to induce secretion of IgA. The latter cytokine had a greater effect compared to IL-10 and could also function in the absence of IL-4 in a dose-dependent fashion. IL-10 was shown to be an IgA switch factor as it was able to induce the production of this isotype by naïve B cells in the absence of IL-4 (318). TGF-β has been widely shown to switch both human and murine B cells to IgA secretion in the absence or presence of IL-4 (319), (69), (320), (321), (322) and (323). This work was carried out using naïve, $sIgA^-$ B cells proving the direct IgA switching effect of TGF-β. These results were consistent with our findings on the protein level.

In conclusion, cytokines that were found able to induce the secretion of certain Ig isotypes in an individual basis included: IL-4 (IgE), IL-10 (IgG4), IFN-γ (IgG1) and TGF-β (IgA).

CHAPTER 4: EFFECT OF CYTOKINES ON IgE PRODUCTION BY HUMAN B CELLS.

4.1 INTRODUCTION.

The maturation of resting B lymphocytes into Ig-secreting cells is a highly regulated phenomenon, thought to be coupled to considerable proliferation, which requires the participation of antigen, T cells and accessory cells acting through cell-cell interactions via specific membrane ligands and through the release of cytokines (324).

During B-cell development and differentiation, cytokines act in autocrine and paracrine fashion. During B-cell ontogeny, IL-7 induces proliferation of B-cell precursors (325), and drives the development of the latter to an immature B cell (326). IL-10 has been shown to be involved in the development of plasma cells from immature B cells (327). IL-4 and IL-13 can switch naïve B cells to IgG4 and IgE production (170), (165), (127) and (111). Abnormal expression of cytokine receptors, aberrant signal transduction and altered cytokine profiles "*in vivo*", can cause functional abnormalities of the B cells (328).

Apart from the cytokines, cell-surface markers play an essential role in the regulation of Ig subclass switching, especially in the case of IgE. As established in Chapter 3, anti-CD40 is a very important factor in the process of IgE production by the human B cells. This soluble monoclonal antibody can substitute for activated T cells or T-cell membranes by providing the survival signal for the B cells "*in vitro*". Hence, anti-CD40 together with IL-4 (at their optimum concentrations), were used as control conditions in our IgE system.

Previous studies, concerning the IgE regulation in both humans and mice, have followed the Th1/Th2 paradigm. Consequently, type 1 cytokines such as IL-2, IFN-γ and TGF-β

have been shown to downregulate the IgE switching process by human B cells (316). (329). (193) and (117). On the other hand, cytokines such as IL-4 (type 2 cytokine). IL-5. IL-6. IL-9 and IL-13 have been shown to upregulate IgE secretion by human and mouse B cells mainly "*in vitro*". These cytokines seem to coordinate host responses against large, extracellular pathogens such as helminths (330). Most of the characteristic features of atopy and asthma, especially IgE synthesis, result from the combined effects of type 2 cytokines and are closely associated with Th2-like cells (331) and (332).

Our results concerning the effects of the cytokines tested on the IL-4-dependent IgE secretion were contradictory with some of the previous findings although the general type 1/type 2 cytokine theory fell into place.

B-cell proliferation experiments were also carried out in parallel with the IgE secretion studies in order to assess the possible correlation between B-cell proliferation and IgE production by the human B cells. For this purpose, linear regression plots (LRP) have been constructed for each cytokine that was found to have an effect on the IgE secretion system "*in vitro*".

In this chapter we attempted to investigate the regulation of different cytokines on the IL-4-dependent IgE switching of the human B cells "*in vitro*".

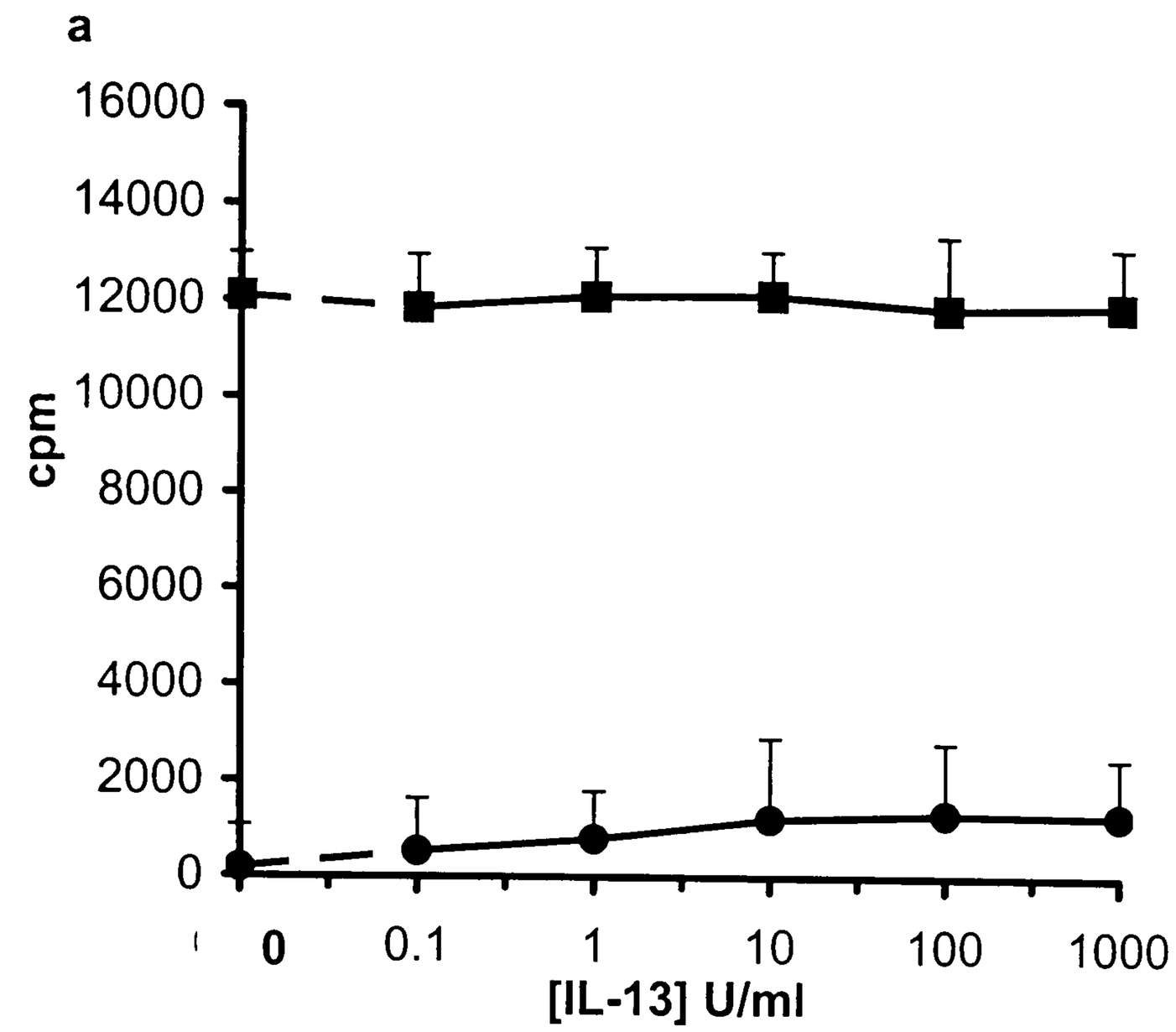
4.2 RESULTS.

4.2.1 Effect of IL-13 on B-cell proliferation and IgE production in the presence or absence of IL-4.

IL-13 was added the B cell cultures at a concentration range from 0-1000 U/ml, in the presence or absence of IL-4. B-cell proliferation was assessed by ^{3}H-Thymidine incorporation after 4 days, while the levels of IgE were measured by the standard IgE sandwich ELISA after 10 days. In figure 4.1 we compared the proliferation of B cells cultured with IL-13 in the presence or absence of IL-4. Both IL-4 and IL-13 stimulated B-cell proliferation (12000cpm and 2000cpm ± SEM respectively). IL-4 was much more potent than IL-13. Similarly, IL-4 induced more IgE than IL-13 alone. No synergy between IL-4 and IL-13 was observed for proliferation or IgE synthesis.

4.2.2 Effect of IL-6 and IL-10 on the IL-4-dependent B-cell proliferation and IgE production.

IL-6 and IL-10 were individually titrated in the B-cell cultures at concentrations ranging from 0-1000 U/ml, together with IL-4 (20 ng/ml) and anti-CD40 (0.5 μg/ml). B-cell proliferation was assessed by ^{3}H-Thymidine incorporation after 4 days of culture, while the levels of IgE were measured by the standard IgE sandwich ELISA after 10 days of culture. The proliferation and IgE switching experiments were repeated at least three times for each cytokine (three donors shown).



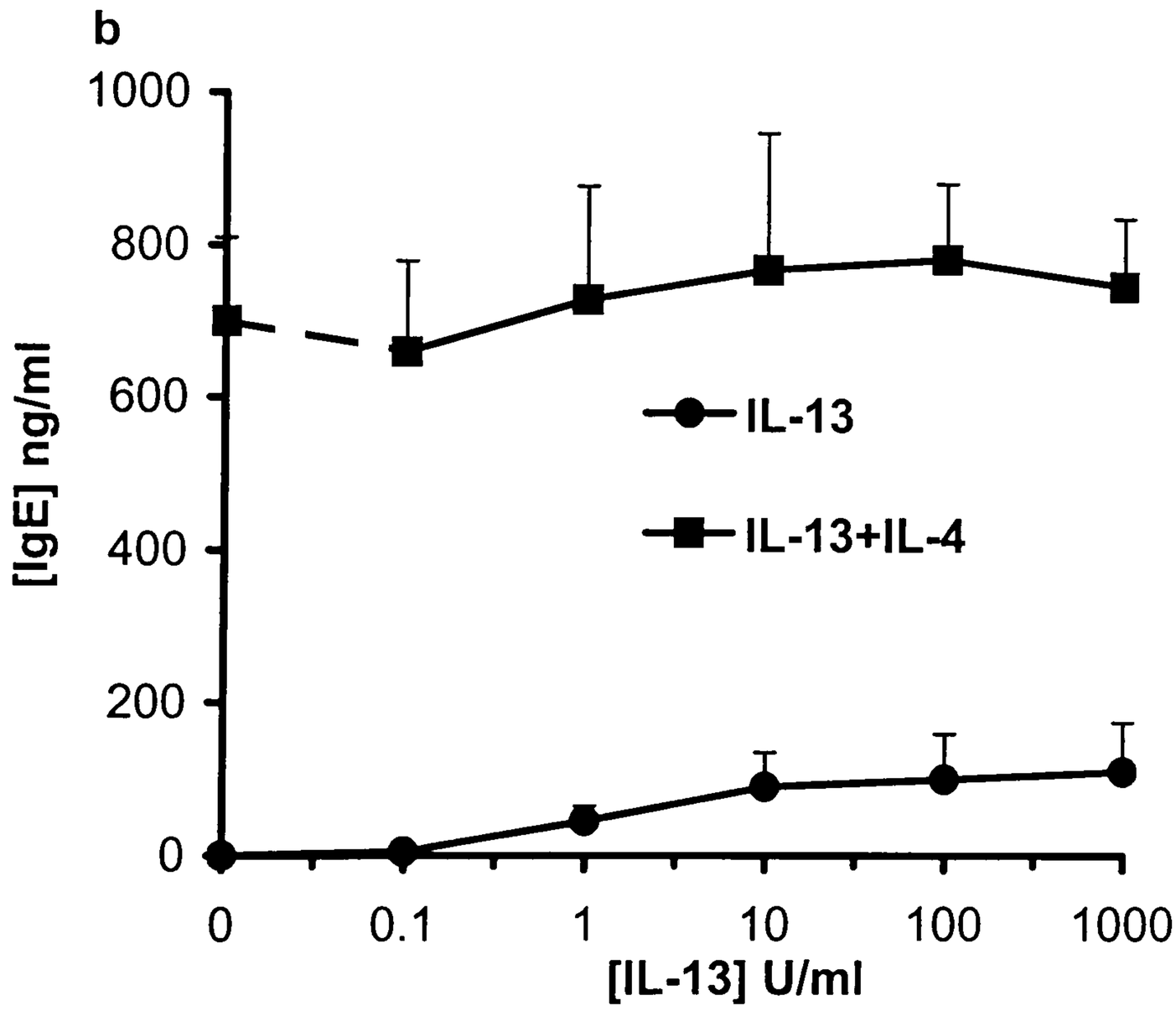


Fig. 4.1: Effect of IL-13 on B-cell proliferation and IgE secretion in the presence or absence of IL-4. Tonsillar B cells were purified by T-cell rosetting and cultured for 4 days in the presence (filled squares) or absence of IL-4 (20 ng/ml) (filled circles) with increasing amounts of IL-13. Anti-CD40 was added at a concentration of 0.5 µg/ml. Proliferation was measured by 3H-Thymidine incorporation after 4 days of culture (a).
Supernatants were collected after a 10 day incubation and the IgE levels measured by ELISA (b). The results are representative of three donors and the standard deviations have been calculated.

Both IL-6 and IL-10 significantly increased IL-4-dependent B cell proliferation and IgE secretion when added at a concentration of 1 ng/ml or more (figures 4.2 and 4.3 respectively). IL-6 increased B-cell proliferation up to 18000cpm and IgE levels to 3000 ng/ml while IL-10 pushed the B-cell proliferation levels to just over 26000cpm while the IgE levels reached levels of 4000 ng/ml. The immunoproliferative and IgE enhancing effect of IL-10 was greater than that of IL-6 (3500 ng/ml and 2500 ng/ml ± SEM respectively).

4.2.3 Effect of IFN-γ, TGF-β, IL-2 and IL-12 on the IL-4-dependent B-cell proliferation and IgE switching.

IFN-γ, TGF-β, IL-2 and IL-12 were individually titrated in the B-cell cultures at concentrations ranging from 0-1000 U/ml, together with IL-4 (20 ng/ml) and anti-CD40 (0.5 μg/ml). B-cell proliferation was assessed by ^{3}H-Thymidine incorporation after 4 days of culture, while the levels of IgE were measured by the standard IgE sandwich ELISA after 10 days of culture. The proliferation and IgE switching experiments were repeated at least three times for each cytokine (three donors shown).

IFN-γ did not affect B-cell proliferation although it inhibited IgE production more than 50% (figure 4.4). TGF-β caused an inhibition of B-cell proliferation and IgE synthesis at the lowest concentration of 0.1 U/ml, while at the highest concentrations (1000 U/ml), IgE production was almost reduced to background levels (figure 4.5). IL-12 had similar effects compared to IFN-γ as it did not affect B-cell proliferation but decreased IgE secretion by over 50% (figure 4.6).

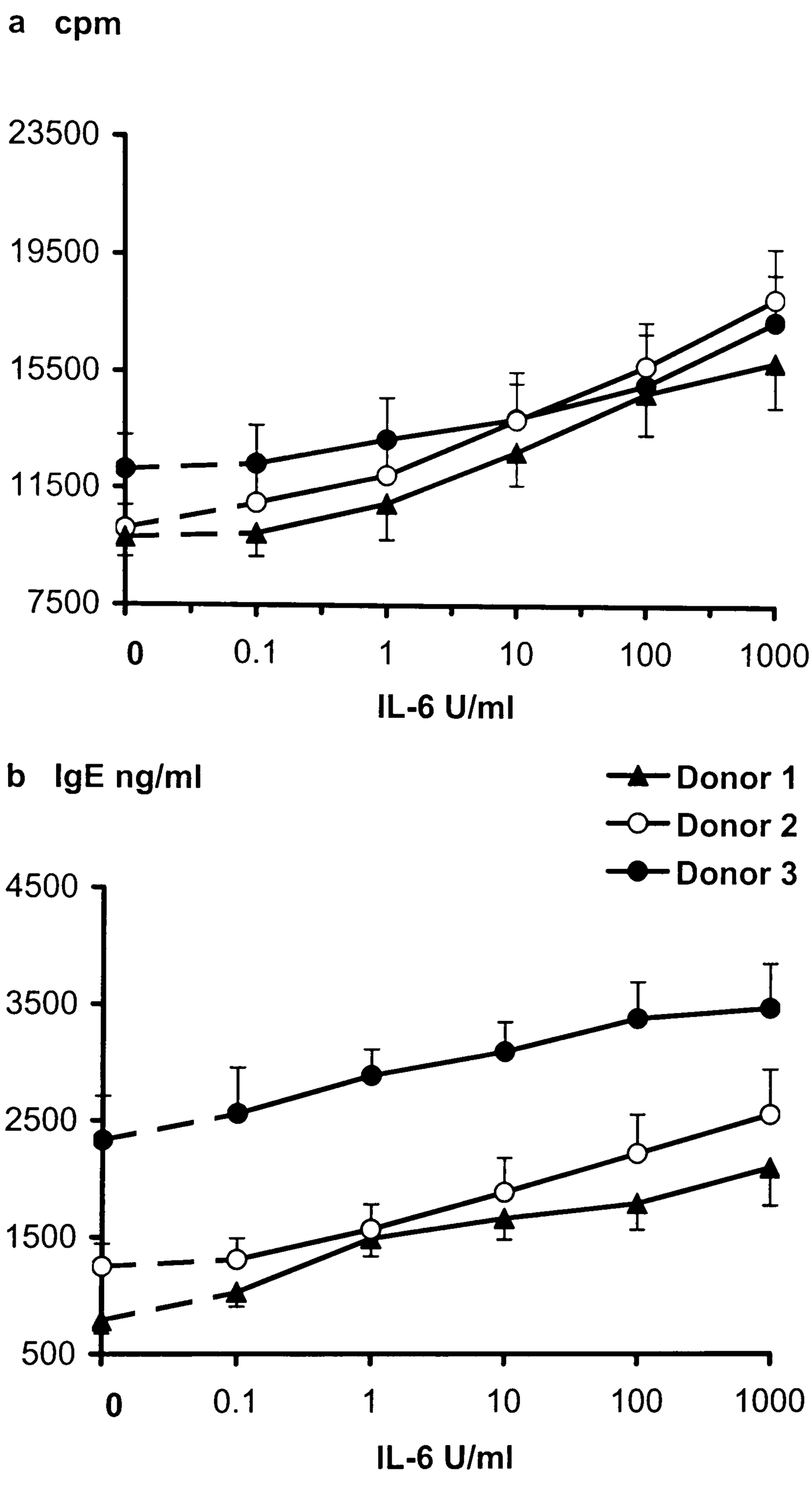


Fig. 4.2: Effect of IL-6 on the IL-4-dependent B-cell proliferation and IgE secretion. Tonsillar B cells from three donors were purified by T-cell rosetting and cultured for 4 days at a concentration of 10^5 cells/well, with IL-4 (20 ng/ml) and anti-CD40 (0.5µg/ml) with increasing amounts of IL-6. Proliferation was determined after 4 days of culture by ^{3}H-Thymidine incorporation (a). Supernatants were collected after 10 days of incubation and the IgE levels were measured by ELISA (b). Standard deviations have been calculated for each individual donor (triplicates).

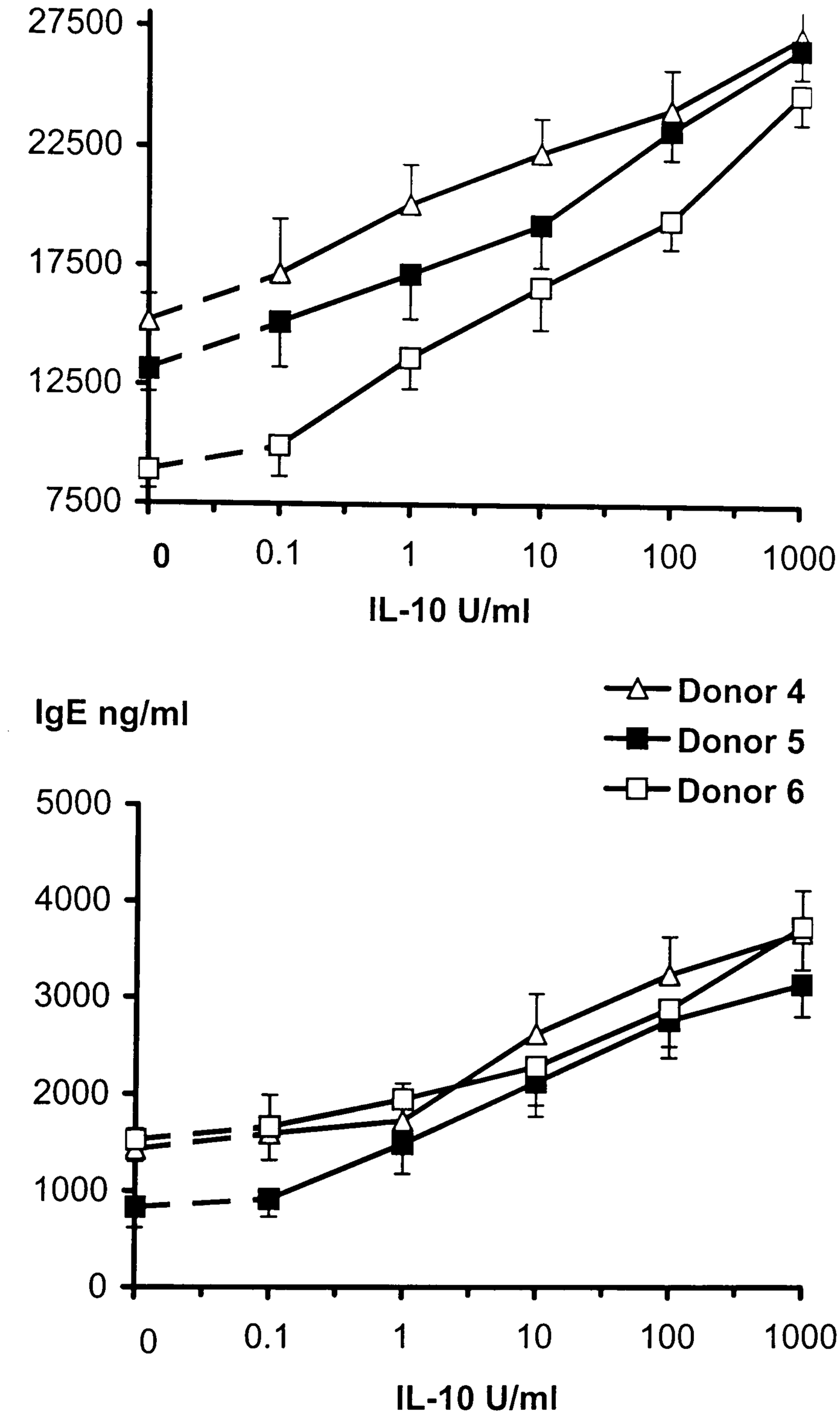


Fig. 4.3: Effect of IL-10 on the IL-4-dependent B-cell proliferation and IgE secretion. Tonsillar B cells from three donors were purified by T-cell rosetting and cultured for 4 days at a concentration of 10^5 cells/well, with IL-4 (20 ng/ml) and anti-CD40 (0.5µg/ml) with increasing amounts of IL-10. Proliferation was determined after 4 days of culture by ^{3}H-Thymidine incorporation (a). Supernatants were collected after 10 days of incubation and the IgE levels were measured by ELISA (b). Standard deviations have been calculated for each individual donor (triplicates).

Interestingly, despite the downregulatory potential of IL-2 on the IL-4-dependent IgE synthesis, the high immunoproliferative effect on the tonsillar B cells was evident (figure 4.7).

4.2.4 Linear Regression Plots (L.R.P)

L.R.P. between B-cell proliferation and IgE secretion were plotted for IL-6 (fig. 4.8a), IL-10 (figure 4.8b), IFN-γ (figure 4.9a), TGF-β (figure 4.9b), IL-12 (figure 4.10a) and IL-2 (figure 4.10b). The correlation coefficient, r, was calculated in order to study the possible relation between B-cell proliferation and IgE secretion levels in response to each of these cytokines. The correlation coefficient of IL-6 and IL-10 was >0.9 which indicated a high positive correlation between proliferation and IgE secretion by the human B cells. A positive correlation was found in the case of TGF-β in which both proliferation levels and IgE production decreased in a dose-dependent fashion. A negative correlation was concluded in the case of IL-2 (increase of B-cell proliferation but drop of IgE levels), while with IL-12 and particularly IFN-γ there was no correlation between the two processes.

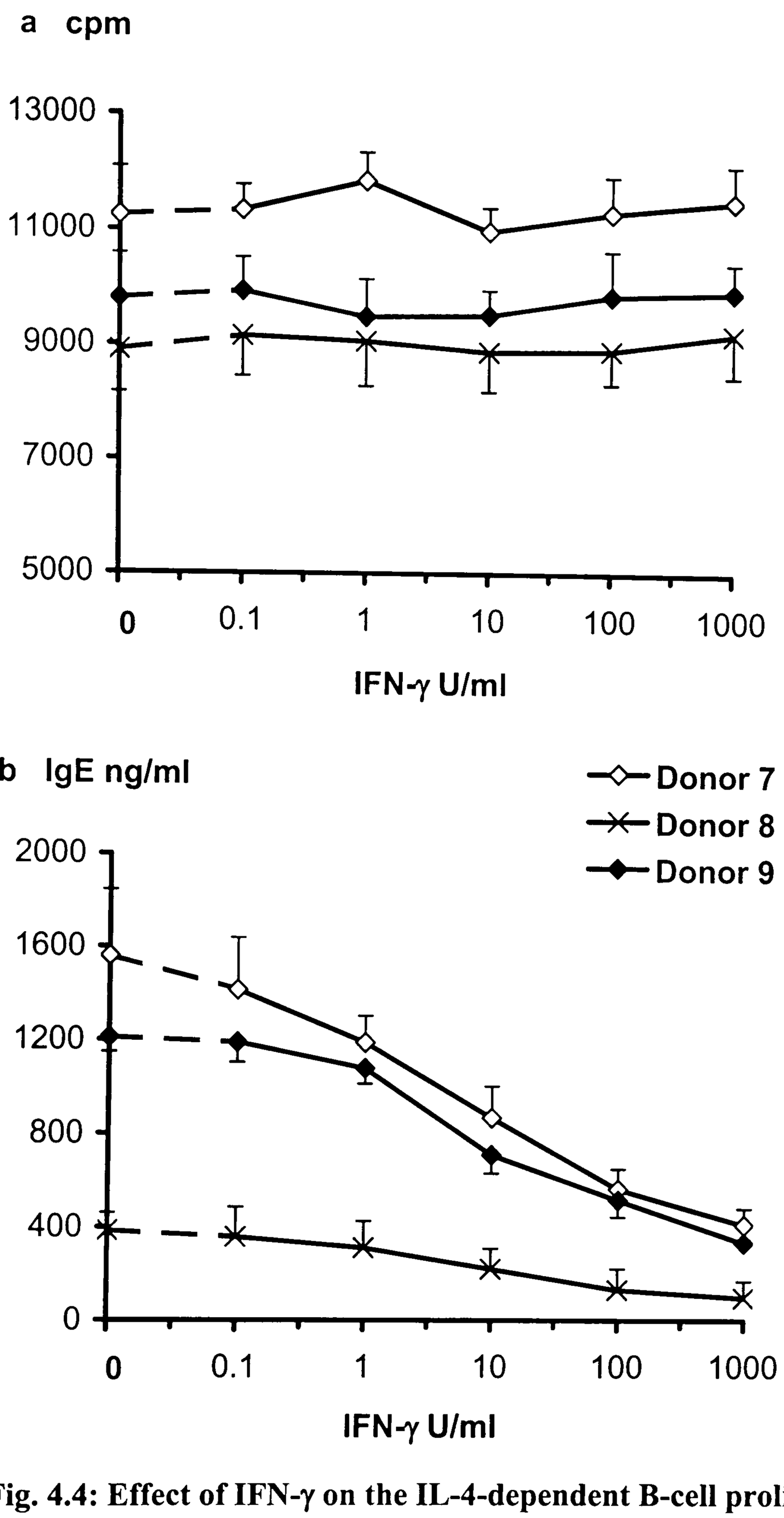


Fig. 4.4: Effect of IFN-γ on the IL-4-dependent B-cell proliferation and IgE secretion. Tonsillar B cells from three donors were purified by T-cell rosetting and cultured for 4 days at a concentration of 10^5 cells/well, with IL-4 (20 ng/ml) and anti-CD40 (0.5μg/ml) with increasing amounts of IFN-γ. Proliferation was determined after 4 days of culture by ^{3}H-Thymidine incorporation (a). Supernatants were collected after 10 days of incubation and the IgE levels were measured by ELISA (b). Standard deviations have been calculated for each individual donor (triplicates).

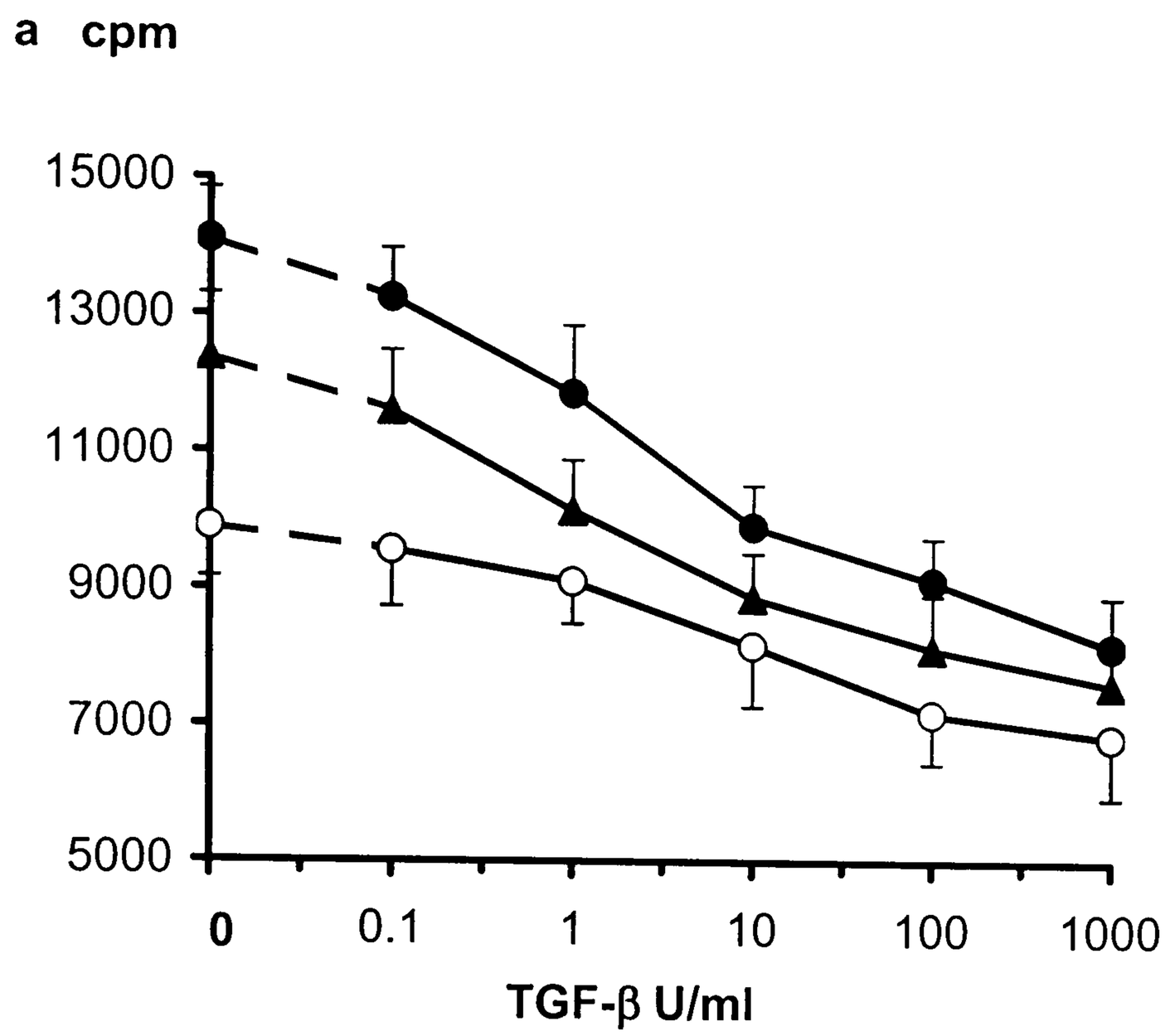


Fig. 4.5: Effect of TGF-β on the IL-4-dependent B-cell proliferation and IgE secretion. Tonsillar B cells from three donors were purified by T-cell rosetting and cultured for 4 days at a concentration of 10^5 cells/well, with IL-4 (20 ng/ml) and anti-CD40 (0.5μg/ml) with increasing amounts of TGF-β. Proliferation was determined after 4 days of culture by ^{3}H-Thymidine incorporation (a). Supernatants were collected after 10 days of incubation and the IgE levels were measured by ELISA (b). Standard deviations have been calculated for each individual donor (triplicates).

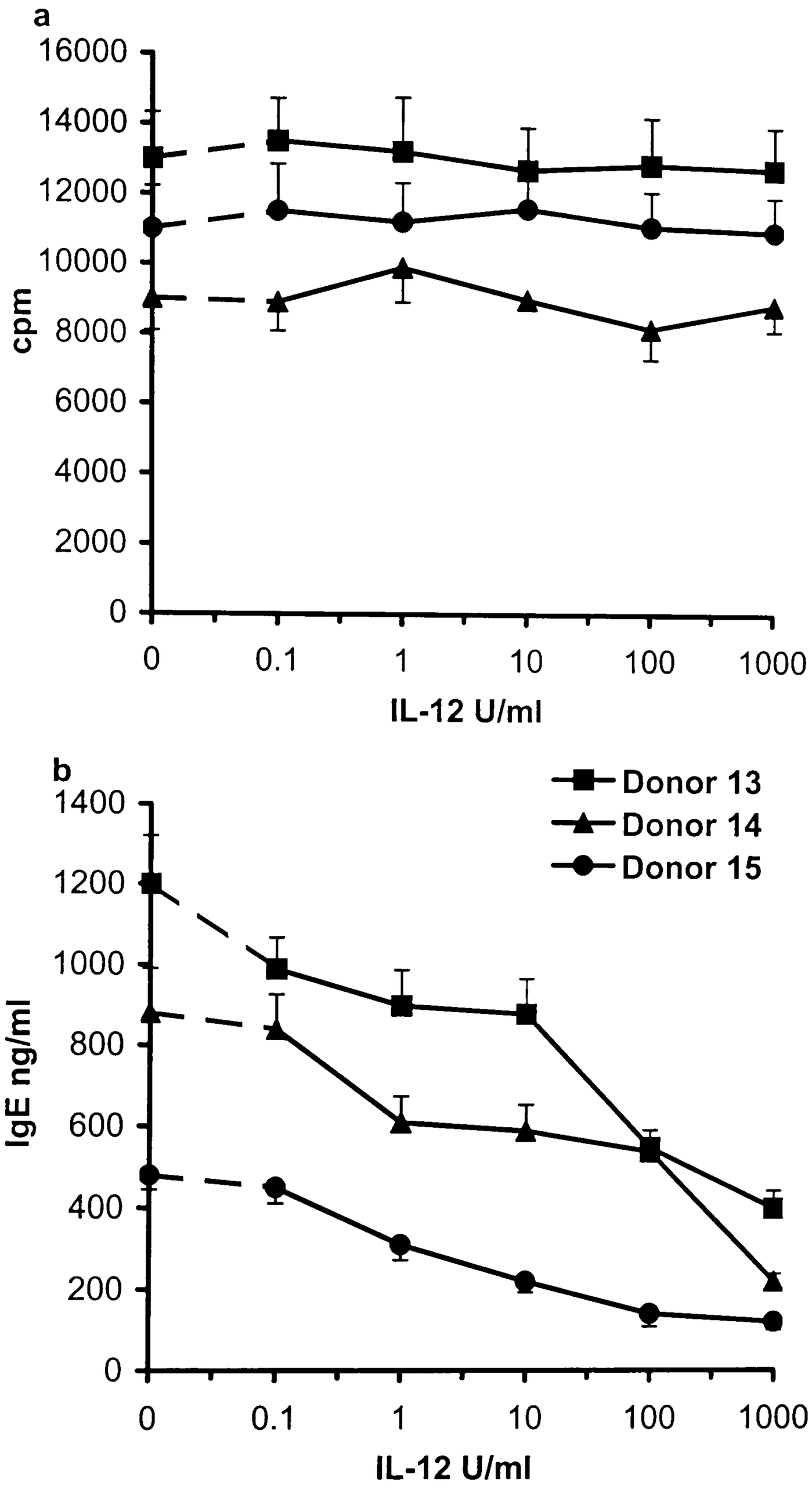


Fig. 4.6: Effect of IL-12 on the IL-4-dependent B-cell proliferation and IgE secretion. Tonsillar B cells from three donors were purified by T-cell rosetting and cultured for 4 days at a concentration of 10^5 cells/well, with IL-4 (20 ng/ml) and anti-CD40 (0.5μg/ml) with increasing amounts of IL-12. Proliferation was determined after 4 days of culture by ^{3}H-Thymidine incorporation (a). Supernatants were collected after 10 days of incubation and the IgE levels were measured by ELISA (b). Standard deviations have been calculated for each individual donor (triplicates).

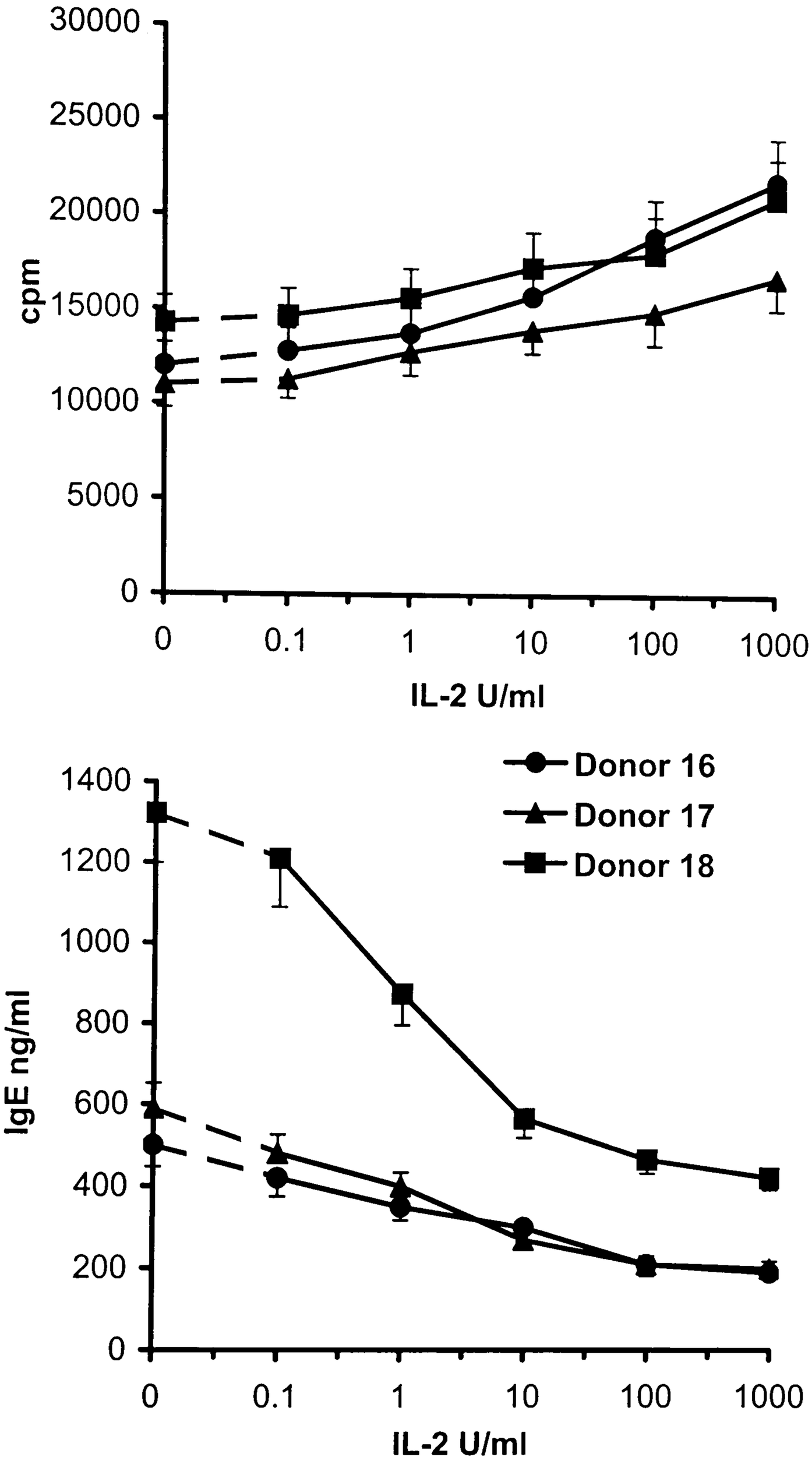


Fig. 4.7: Effect of IL-2 on the IL-4-dependent Bcell proliferation and IgE secretion. Tonsillar B cells from three donors were purified by T-cell rosetting and cultured for 4 days at a concentration of 10^5 cells/well, with IL-4 (20 ng/ml) and anti-CD40 (0.5μg/ml) with increasing amounts of IL-2. Proliferation was determined after 4 days of culture by ^{3}H-Thymidine incorporation (a). Supernatants were collected after 10 days of incubation and the IgE levels were measured by ELISA (b). Standard deviations have been calculated for each individual donor (triplicates).

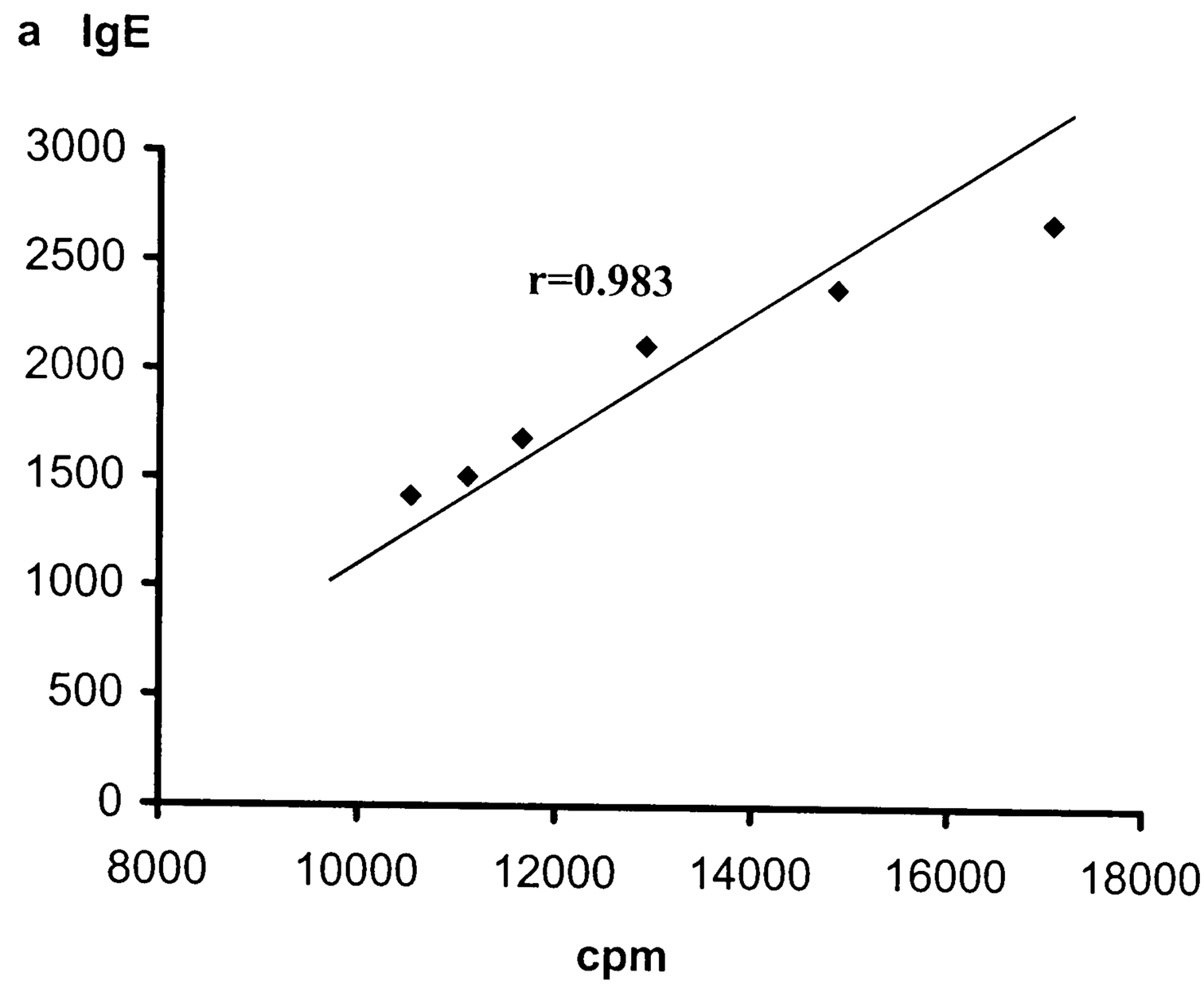


Fig. 4.8: Linear regression plots (LRP). LRPs were constructed based on the effects of IL-6 (a) and IL-10 (b) on B cell proliferation and IgE secretion. The average of three donors was taken and the best fit line was drawn. The possible correlation between the two processes was assessed by calculating the correlation coefficient, r.

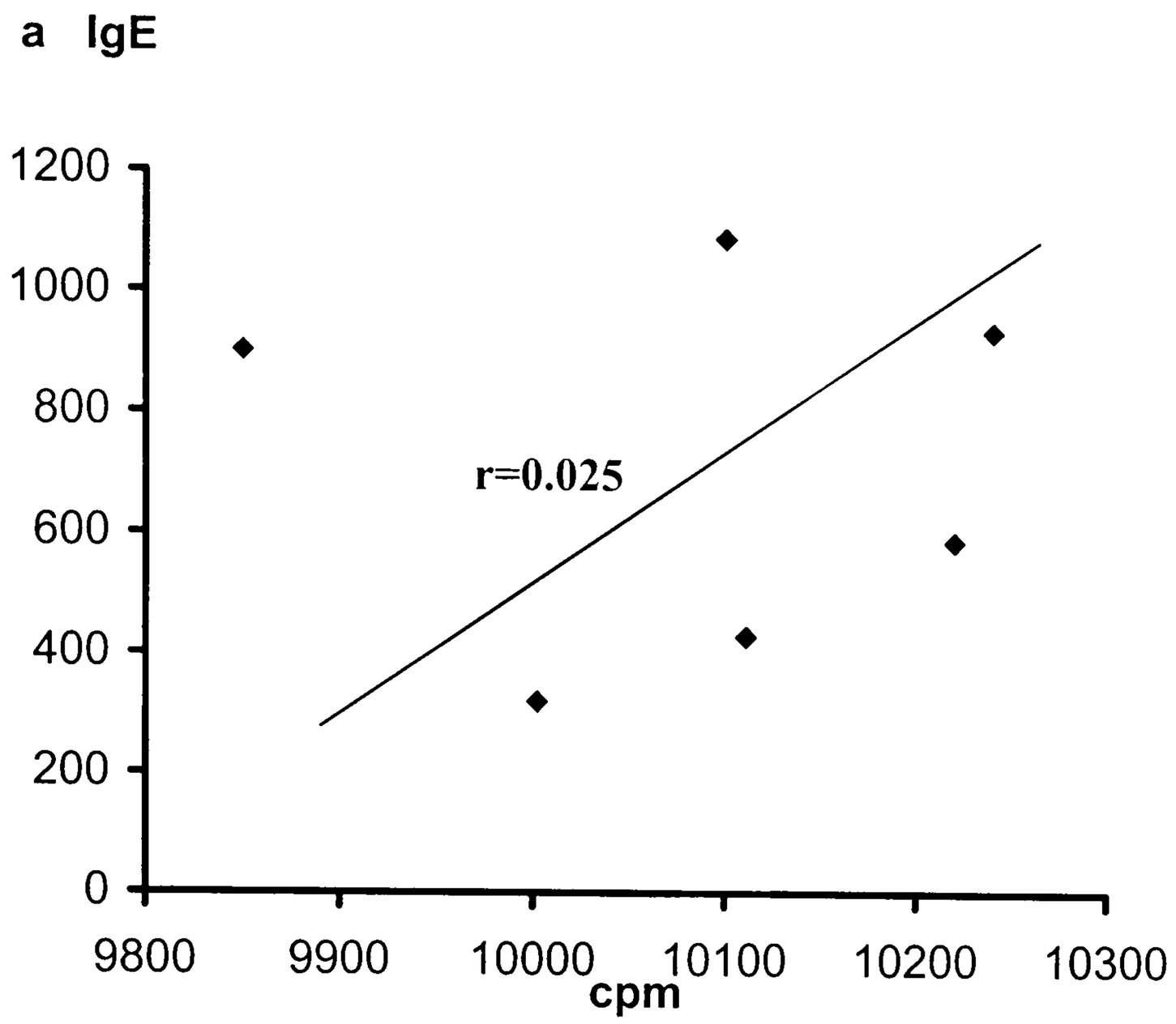
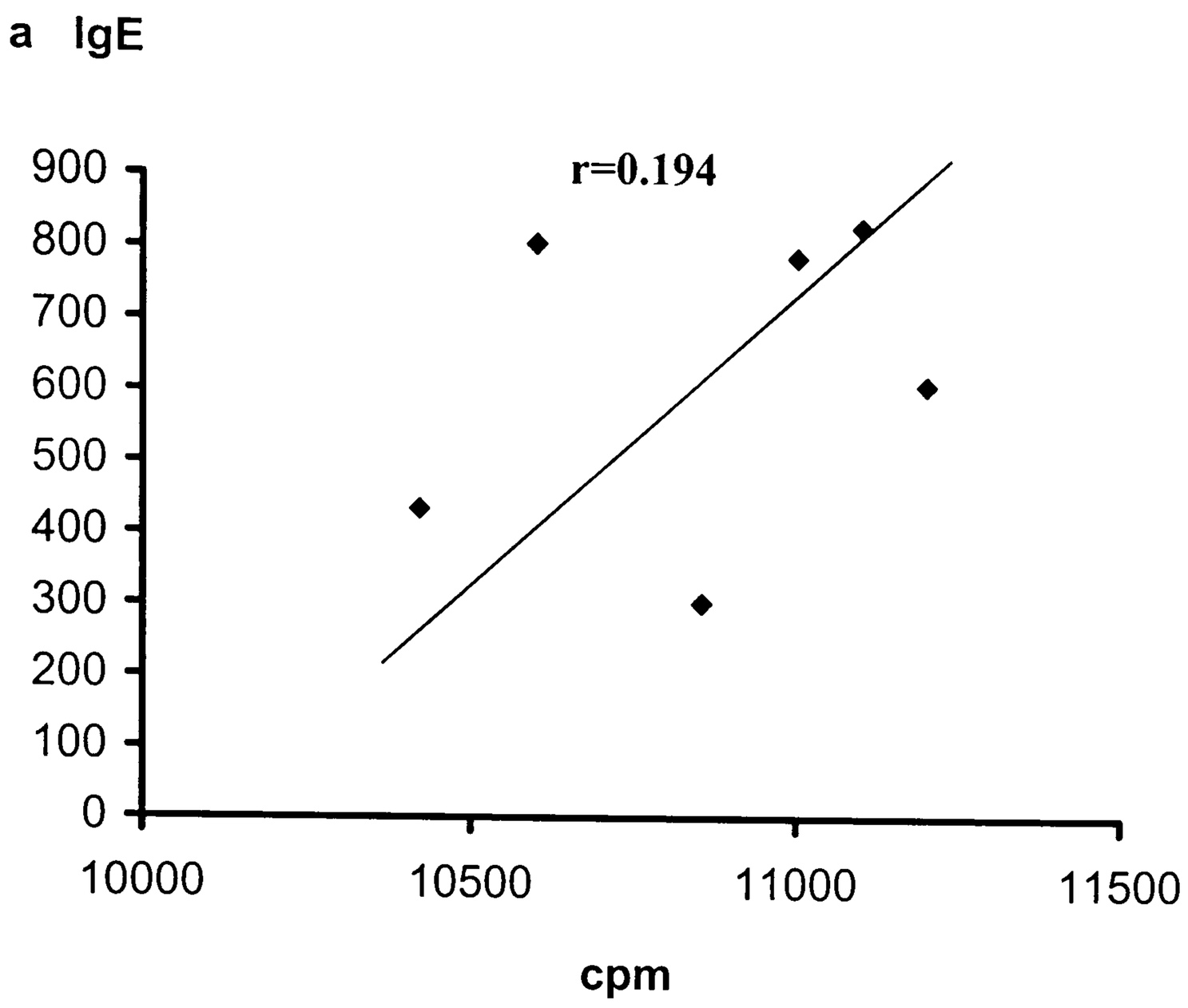


Fig. 4.9: Linear regression plots (LRP). LRPs were constructed based on the effects of IFN-γ (a) and TGF-β (b) on B cell proliferation and IgE secretion. The average of three donors was taken and the best fit line was drawn. The possible correlation between the two processes was assessed by calculating the correlation coefficient, r.



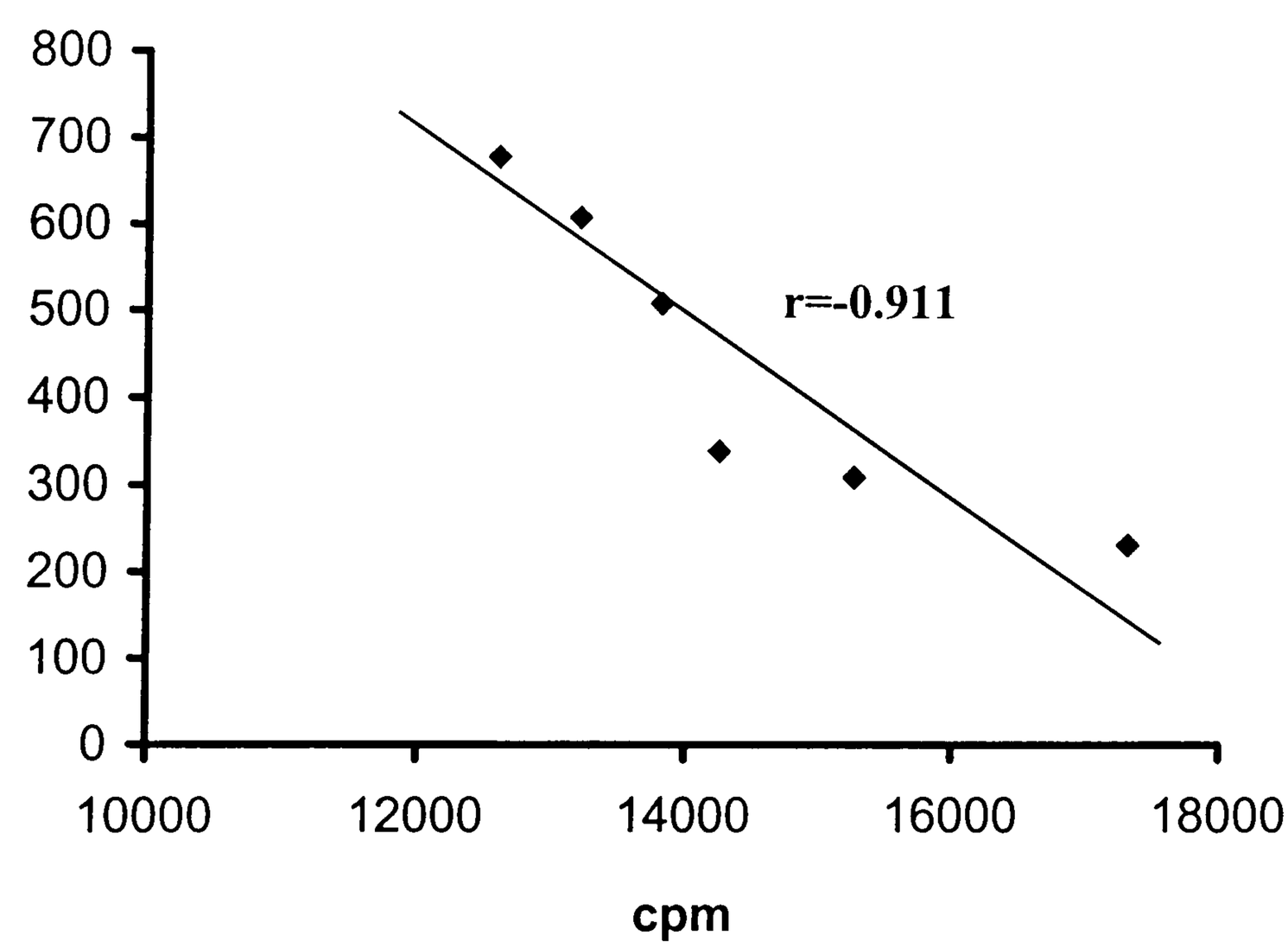


Fig. 4.10: Linear regression plots (LRP). LRPs were constructed based on the effects of IL-12 (a) and IL-2 (b) on B cell proliferation and IgE secretion. The average of three donors was taken and the best fit line was drawn. The possible correlation between the two processes was assessed by calculating the correlation coefficient, r.

4.2.5 Effect of IL-5, IL-7 and IL-9 on IL-4-dependent B-cell proliferation and IgE production.

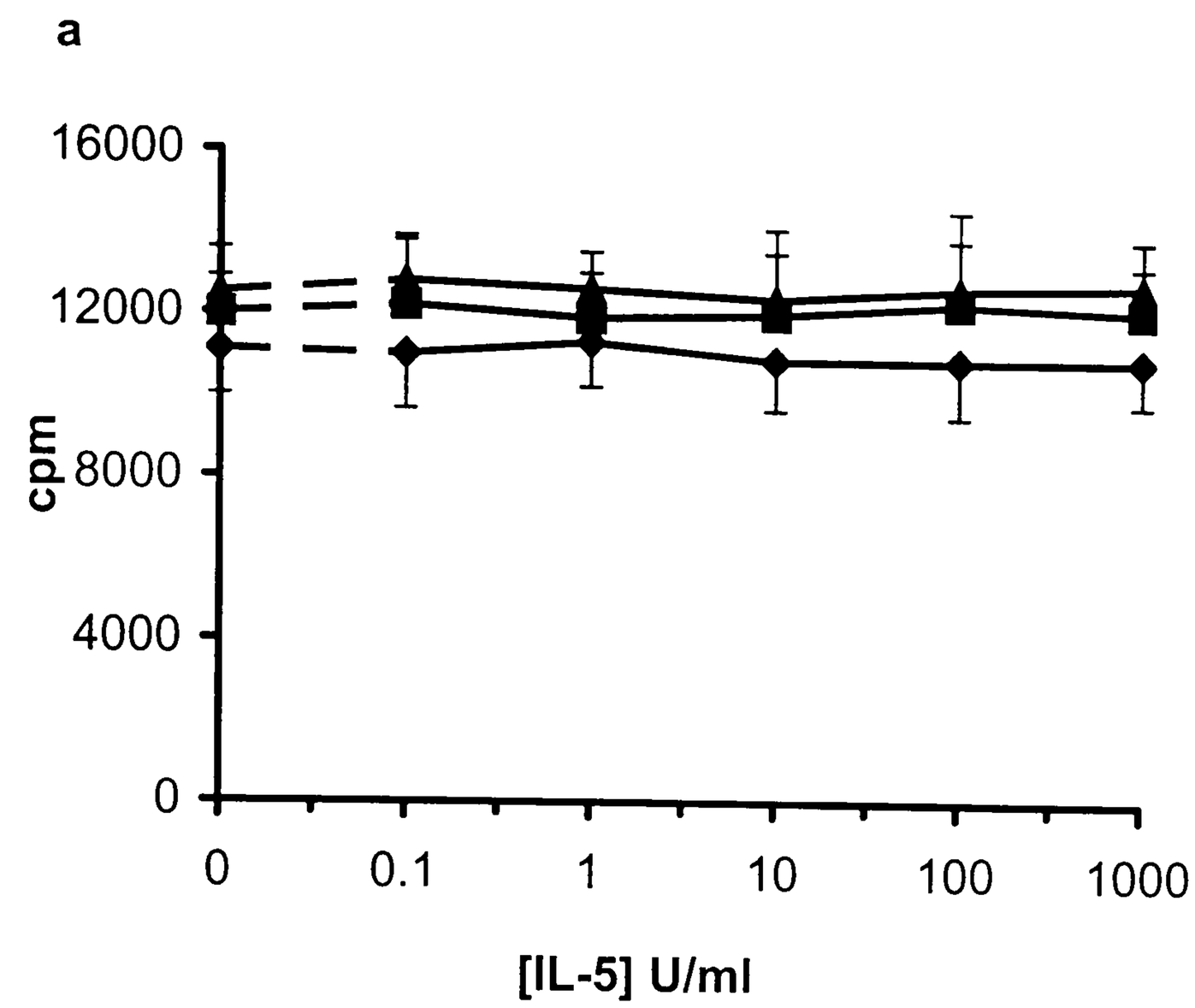
IL-5, IL-7 and IL-9 were individually titrated in the B-cell cultures at concentrations ranging from 0-1000 U/ml, together with IL-4 (20 ng/ml) and anti-CD40 (0.5 μg/ml). B-cell proliferation was assessed by ^{3}H-Thymidine incorporation after 4 days of culture, while the levels of IgE were measured by the standard IgE sandwich ELISA after 10 days of culture. The proliferation and IgE switching experiments were repeated at least three times for each cytokine. Three donors are shown for each cytokine and the standard deviations have been calculated (triplicates). Neither IL-5 nor IL-7 nor IL-9 had an effect on IL-4-dependent B cell proliferation and IgE secretion by the human B cells "*in vitro*" (figures 4.11, 4.12 and 4.13 respectively).

4.2.6 Possible effects of the B-cell purification methods on the IL-4-dependent B-cell proliferation and IgE production.

Having established the effects of the various exogenous cytokines on the IL-4-dependent B-cell proliferation and IgE production, we wanted to investigate whether the B-cell purification method used could add to or distort these effects. For this purpose two sets of B-cell cultures were prepared. In the first one, B cells were purified by T-cell rosetting while in the second one B cells were positively selected by CD19+ dynabeads from the tonsillar cell suspension. Both sets of cells were cultured with IL-4 (20 ng/ml), anti-CD40 (0.5 μg/ml) and titrated amounts of one exogenous cytokine (IL-6, IL-10, IFN-γ, TGF-β, IL-2 or IL-12). The concentration of the exogenous cytokines ranged

from: 0-1000 U/ml. B-cell proliferation was assessed by ^{3}H-Thymidine incorporation after 4 days in culture, while the levels of IgE were measured by the standard IgE sandwich ELISA after 10 days of culturing. The proliferation and IgE switching experiments were repeated at least three times for each cytokine (the average of each donor is shown).

As shown on figures 4.14 and 4.15, the B-cell purification method did not have an effect on the general trend of each cytokine on the IL-4-dependent B-cell proliferation and IgE secretion. Consequently, IL-6 and IL-10, both enhanced IL-4-dependent B-cell proliferation and IgE secretion using either purification methods while IL-12 and IFN-γ did not affect the levels of B cell proliferation. With TGF-β, B-cell proliferation, while with IL-2 there was a dose-dependent increase in proliferation. The suppressive effects of IFN-γ, TGF-β, IL-12 and IL-2 on IgE secretion were consistent and independent of the purification method used. It`s worth noting that positively selected B cells had higher proliferation levels compared to the B cells purified by T-cell rosetting, while the reverse occurred concerning IgE switching and secretion in the B-cell cultures.



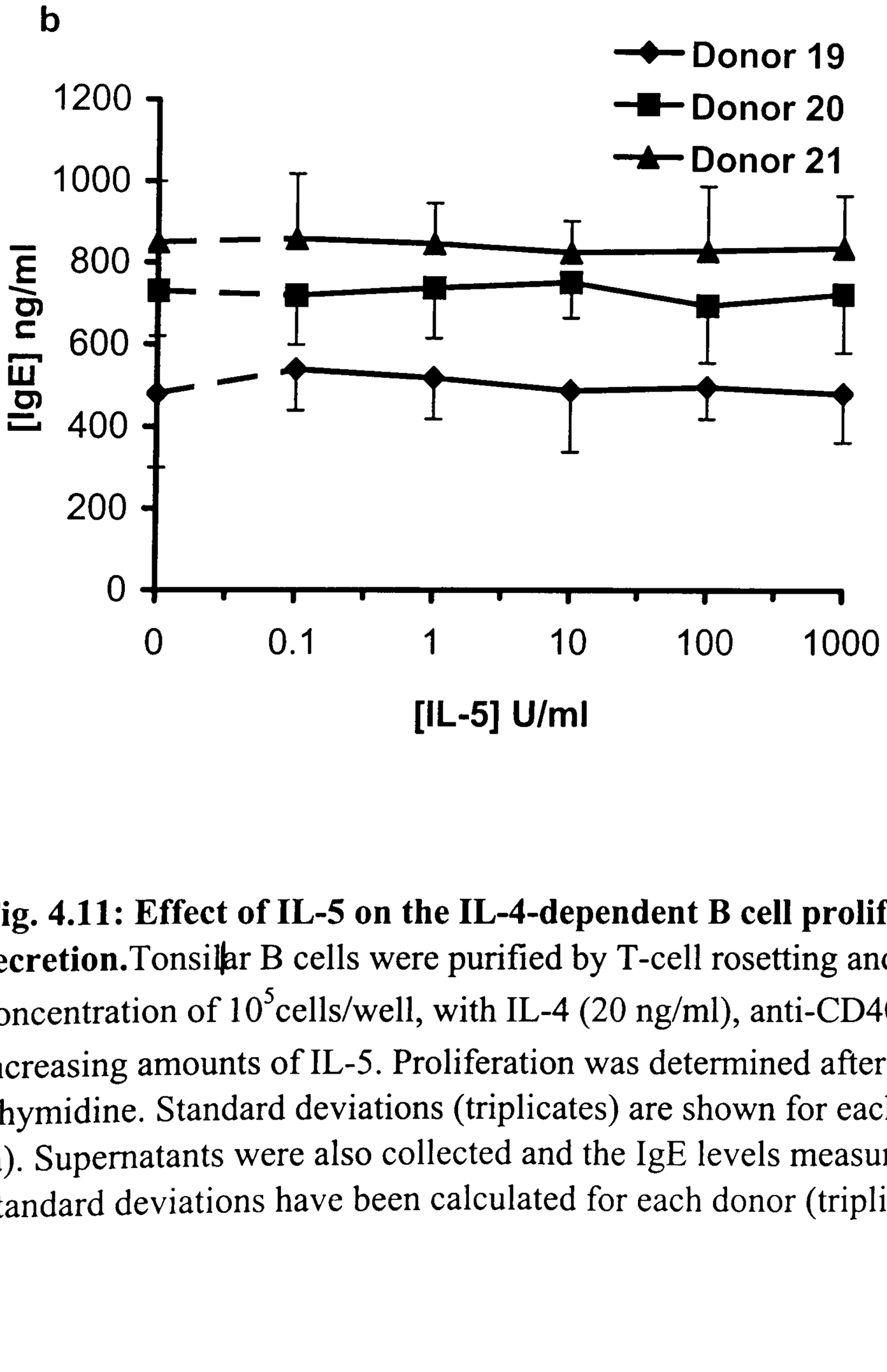


Fig. 4.11: Effect of IL-5 on the IL-4-dependent B cell proliferation and IgE secretion. Tonsillar B cells were purified by T-cell rosetting and cultured at a concentration of 10^5 cells/well, with IL-4 (20 ng/ml), anti-CD40 (0.5 μg/ml) and increasing amounts of IL-5. Proliferation was determined after 4 days by ^{3}H-Thymidine. Standard deviations (triplicates) are shown for each individual donor (a). Supernatants were also collected and the IgE levels measured by ELISA. Standard deviations have been calculated for each donor (triplicates) (b).

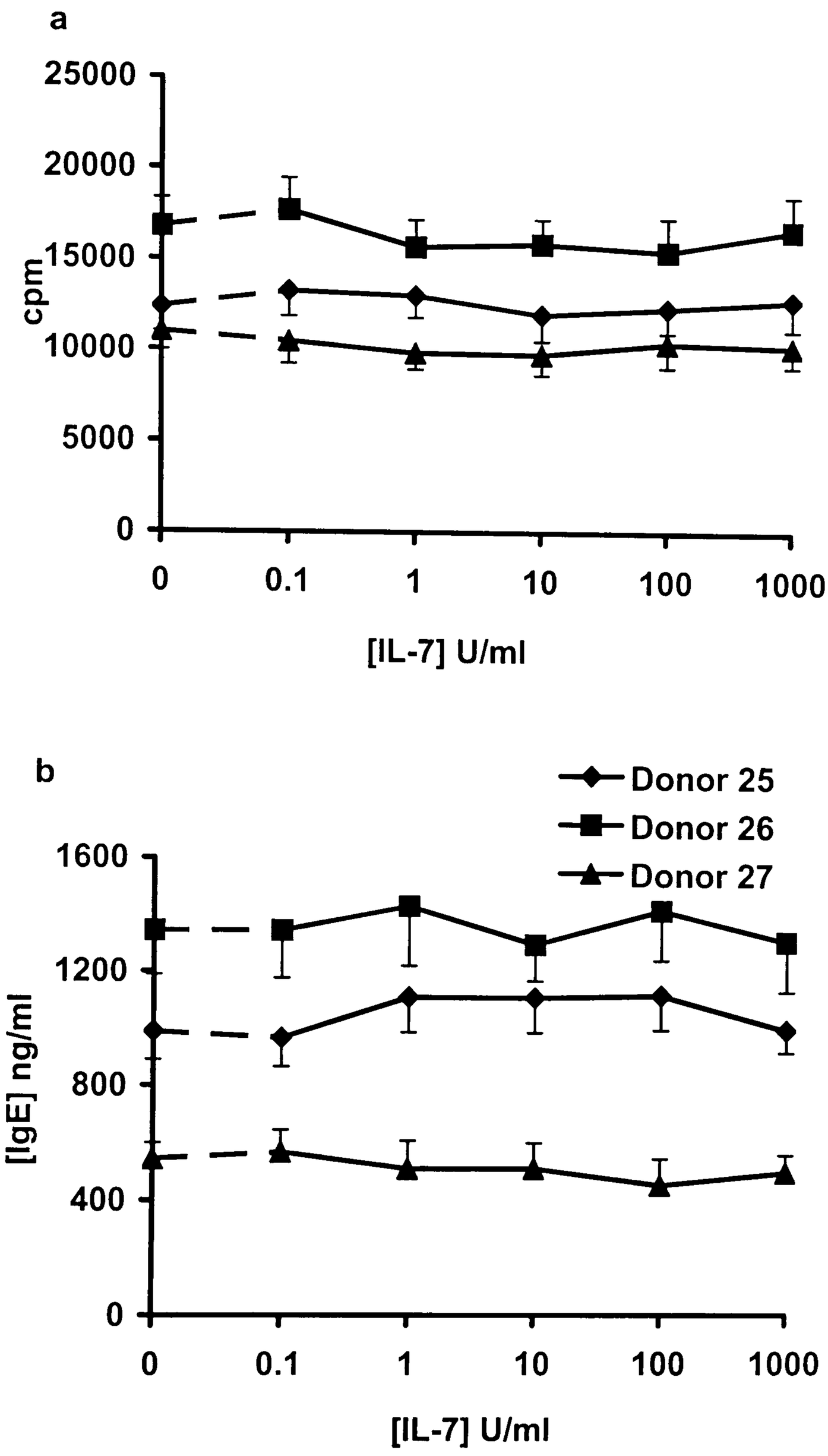


Fig. 4.12: Effect of IL-7 on the IL-4-dependent B cell proliferation and IgE secretion. Tonsillar B cells were purified by T-cell rosetting and cultured at a concentration of 10^5 cells/well, with IL-4 (20 ng/ml), anti-CD40 (0.5 μg/ml) and increasing amounts of IL-7. Proliferation was determined after 4 days by ^{3}H-Thymidine. Standard deviations (triplicates) are shown for each individual donor (a). Supernatants were also collected and the IgE levels measured by ELISA. Standard deviations have been calculated for each donor (triplicates) (b).

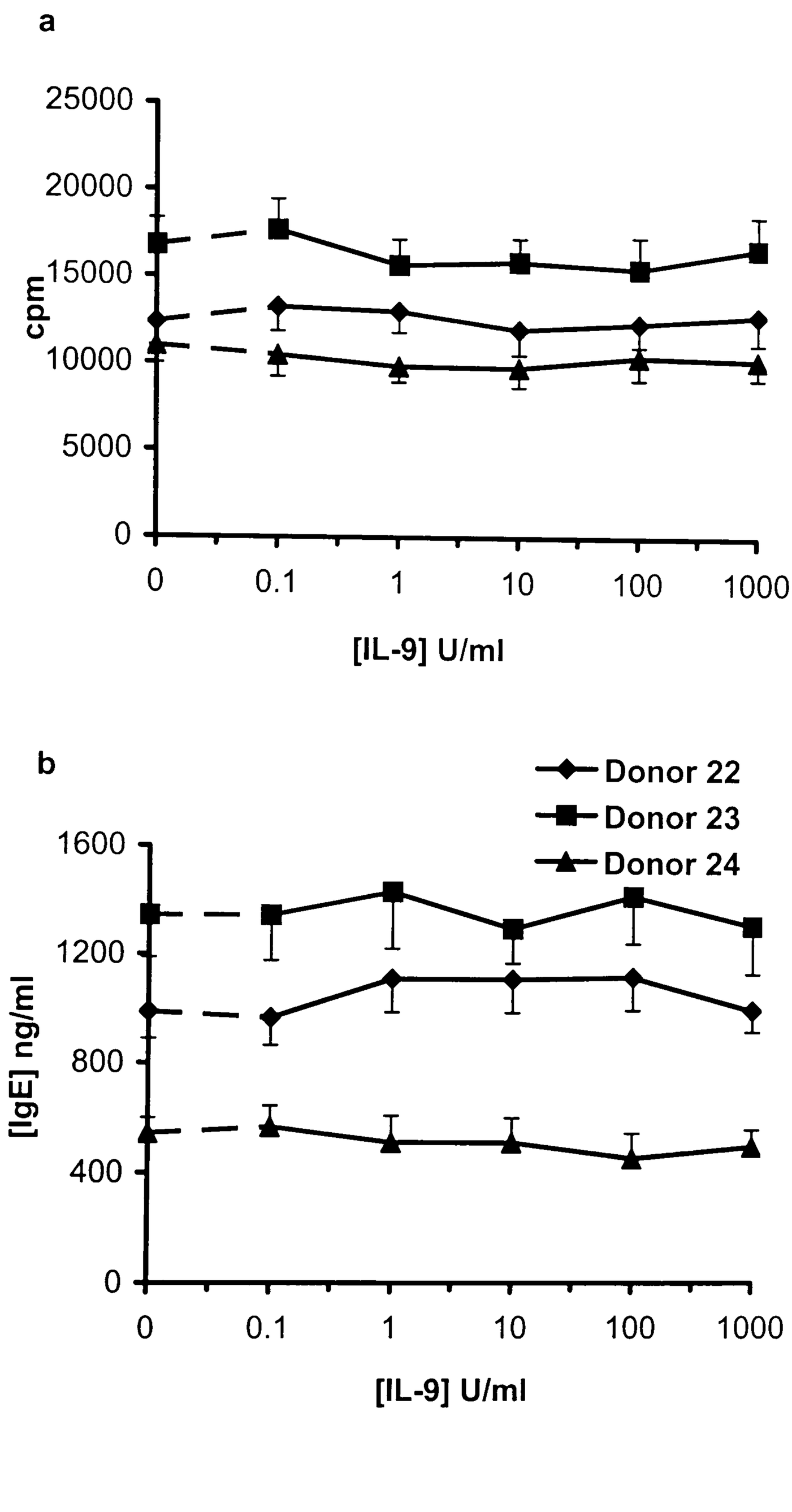


Fig. 4.13: Effect of IL-9 on the IL-4-dependent B cell proliferation and IgE secretion. Tonsillar B cells were purified by T-cell rosetting and cultured at a concentration of 10^5 cells/well, with IL-4 (20 ng/ml), anti-CD40 (0.5 μg/ml) and increasing amounts of IL-9. Proliferation was determined after 4 days by ^{3}H-Thymidine. Standard deviations (triplicates) are shown for each individual donor (a). Supernatants were also collected and the IgE levels measured by ELISA. Standard deviations have been calculated for each donor (triplicates) (b).

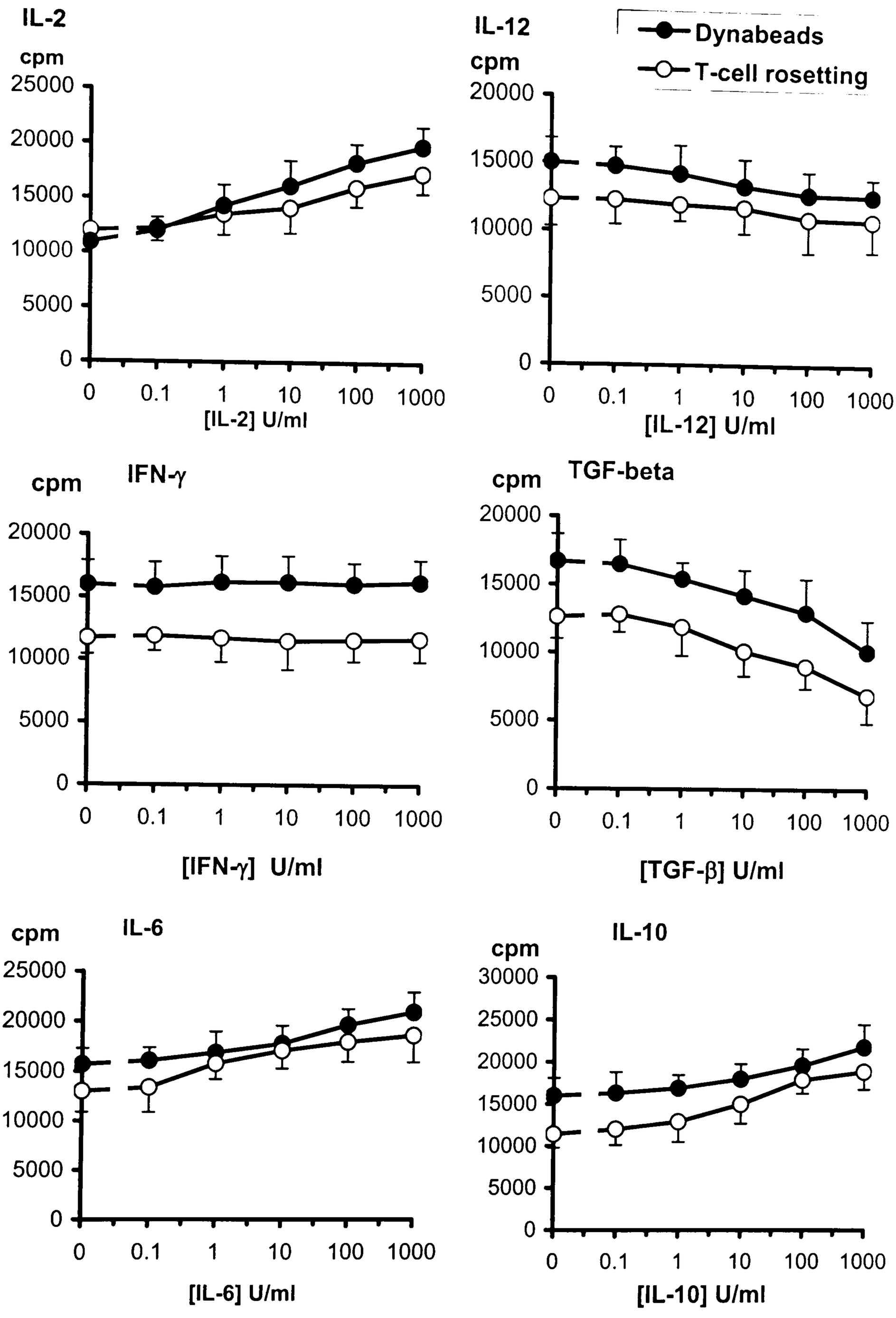


Fig. 4.14: Effect of purification method on B-cell proliferation. Tonsillar B cells from three donors were purified by T-cell rosetting or by CD19 dynabeads (positive selection) and cultured for 4 days at standard conditions, against increasing amounts of each exogenous cytokine. B-cell proliferation was measured by incorporation of ^{3}H-Thymidine after 4 days of culture. The mean values of the three donors were noted. Standard errors are shown.

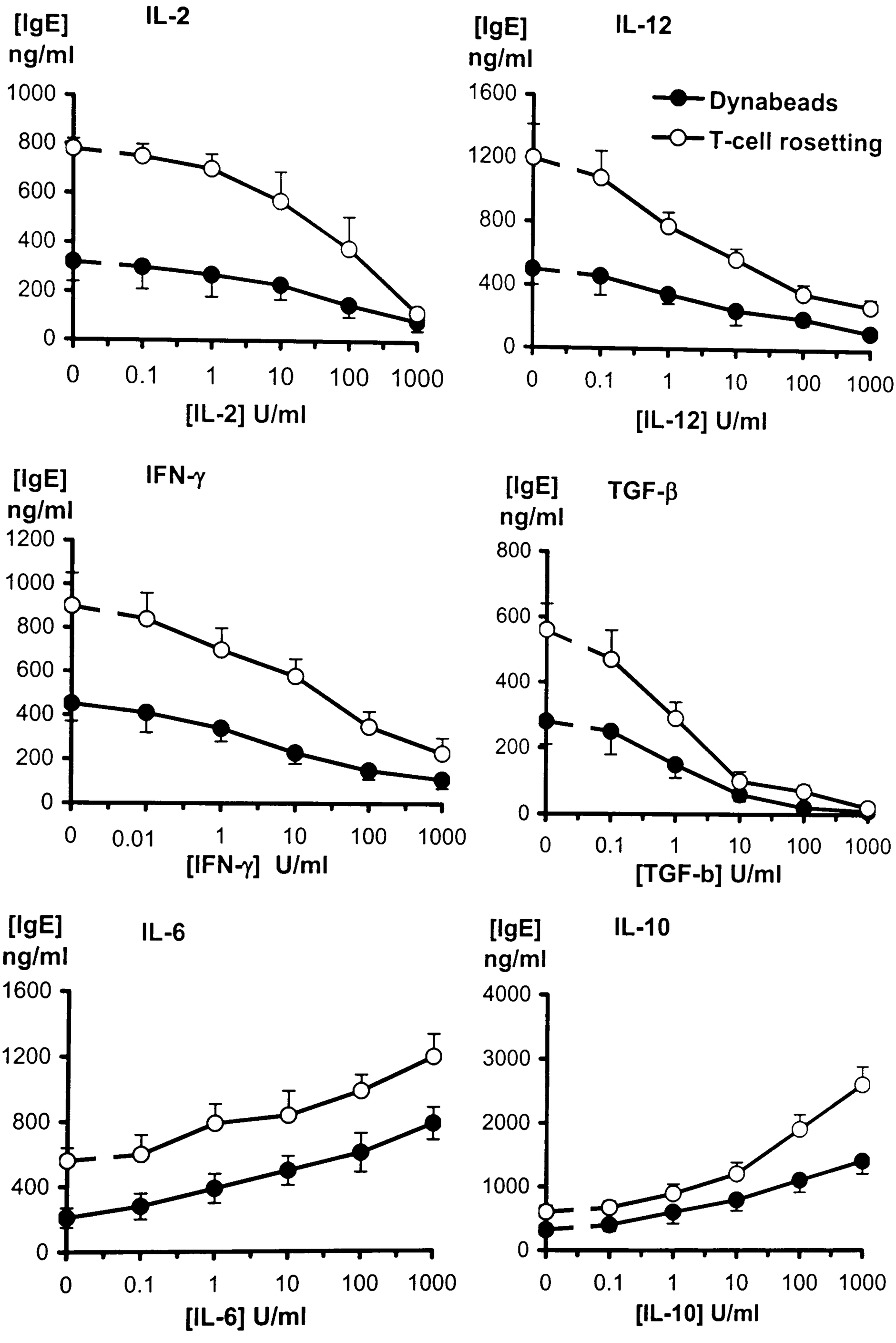


Fig.4.15: Effect of purification method on IgE secretion by human B cells. Tonsilar B cells from three donors were purified by T-cell rosetting or by CD19 dynabeads (positive selection) and cultured for 10 days at standard conditions, against increased amounts of each exogenous cytokine. The IgE levels were measured by ELISA in the supernatants and the means of the three donors are presented. Standard errors are shown.

4.2.7 Effect of IL-18 on IL-4-dependent B-cell proliferation and IgE secretion. Possible synergism with IL-12.

Tonsillar B cells were purified by T-cell rosetting and cultured at standard conditions of IL-4 (20 ng/ml) and anti-CD40 (0.5 μg/ml) (Control). Increasing amounts of IL-18 were titrated in the cultures at concentrations ranging from 0-1000 U/ml. After 4 days the cultures were pulsed with ^{3}H-Thymidine, incubated for a further 6-18 hours and subsequently the counts were measured with a beta-counter. B-cell proliferation was directly related to the incorporation of ^{3}H-Thymidine. After a 10 day culture period, supernatants were obtained and the IgE levels measured by ELISA. IL-4-dependent B-cell proliferation was unaffected by IL-18 while at the higher concentrations of the latter cytokine (100 and 1000 U/ml), IL-4-dependent IgE secretion was enhanced (figure 4.16a).

Using the same donor, B cells were also cultured at standard conditions but under the presence of both IL-18 and IL-12 at a concentration of 100 U/ml. Although IL-18 was found to enhance IL-4-dependent IgE secretion at 100 U/ml, addition of IL-12 brought IgE production down to control levels (figure 4.16b). This experiment is a representative of two donors. The standard deviations for the donor shown were calculated (triplicates).

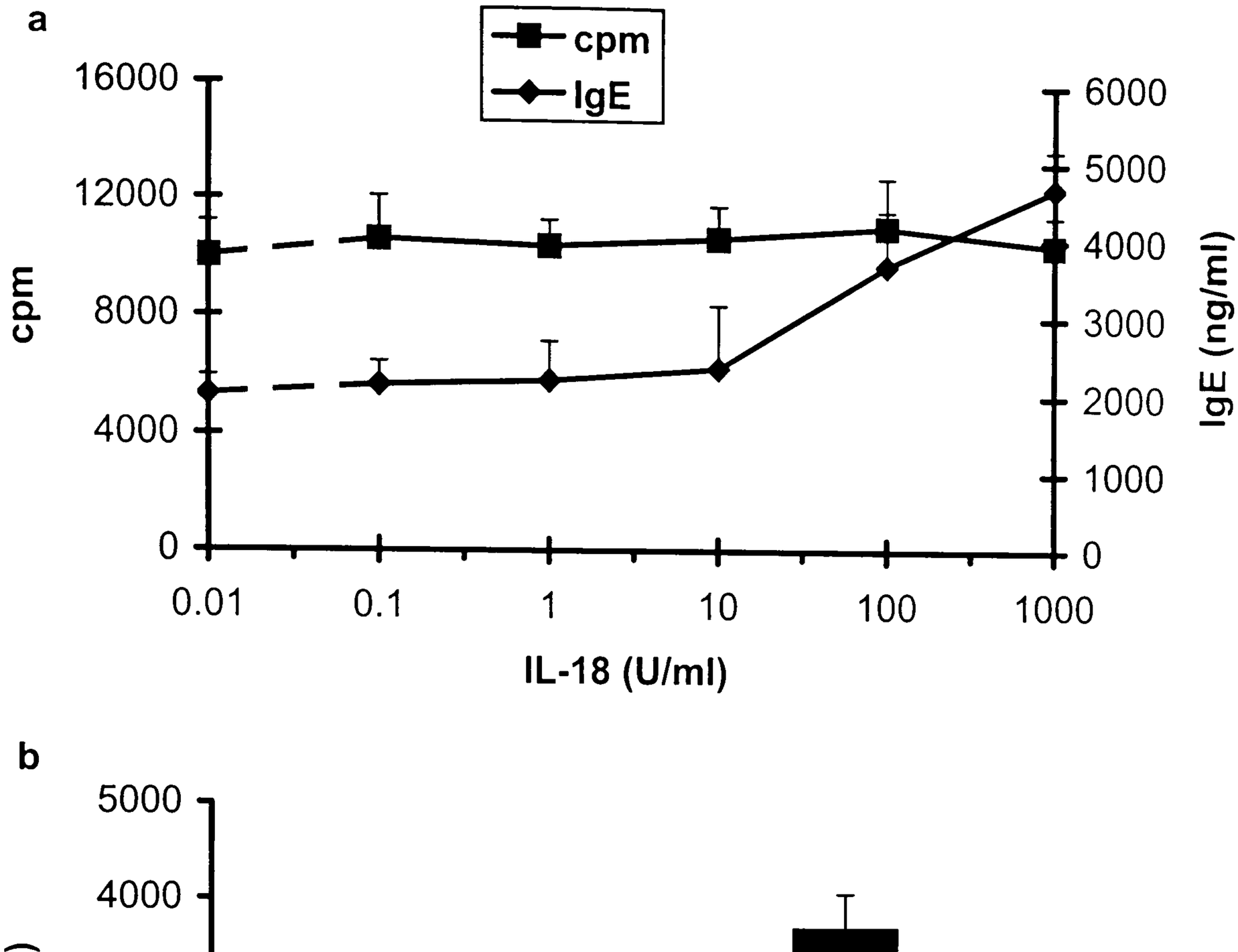


Fig. 4.16a: Effect of IL-18 on the IL-4-dependent B-cell proliferation and IgE secretion. Tonsillar B cells from were purified by T-cell rosetting and cultured for 4 days at a concentration of 10^5 cells/well, with IL-4 (20 ng/ml) and anti-CD40 (0.5 μg/ml) with increasing amounts of IL-18. Proliferation was determined after 4 days of culture by ^{3}H-Thymidine incorporation. Supernatants were collected after 10 days of incubation and the IgE levels were measured by ELISA. These results are the mean of two donors and the standard deviations have been calculated (triplicates).

Fig. 4.16b: Possible synergistic effect of IL-18 and IL-12 on IgE secretion. B cells from were purified by T-cell rosetting and cultured for 4 days at a concentration of 10^5cells/well, with IL-4 (20 ng/ml) and anti-CD40 (0.5 μg/ml), in the presence or absence of IL-18 (100 U/ml) and IL-12 (100 U/ml) or both. After 10 day incubation the IgE levels in the supernatants were measured by ELISA. These results are the mean of two donors and the standard deviations have been calculated (triplicates).

4.3 DISCUSSION.

In this study we investigated the effects of different exogenous cytokines on the IL-4-dependent IgE switching. IL-13 did not have a synergistic effect with IL-4 and on its own was found to mimic the effects of IL-4 although not the extent of the latter cytokine. IL-13 was able to switch B cells to IgE secretion in the absence of IL-4, in a dose-dependent manner, although it was 10-fold less compared to the levels of IgE when IL-4 was present together with IL-13. This finding was probably related to the lower immunoproliferative activity of IL-13 on B cells compared to IL-4. These results suggest the similar biological nature of the two cytokines but at the same time underlines their different potentiating effects in IgE switching "in vitro". The lower B cell response to IL-13 was also shown by Kimata *et al.*, (1995) and Kohler *et al.*, (1994) (333) and (334). The latter group demonstrated activation of STAT 6 by IL-13, a signalling molecule also activated in the IL-4 pathway. The presence of the extra β-subunit in the IL-13 receptor probably accounts for the 10-fold lower proliferation and IgE secretion by the B cells "*in vitro*" (146).

In our study, addition of IFN- γ significantly reduced the IL-4-dependent IgE secretion in the B-cell cultures while the proliferation of the B cells was independent of the concentration of the cytokine. This probably indicates that the suppressive effect of IFN-γ on IgE switching is direct, via some specific molecular pathway. This fact is supported by the ability of IFN-γ to switch human B cells to IgG1 secretion "*in vitro*" (as shown in Chapter 3).

Maggi *et al.* (1989), showed a synergistic effect of IL-6 and IL-4 on proliferation and IgE secretion by both human and murine B cells (143). In this study tonsilar B-cell proliferation increased significantly with increasing IL-6 concentration. Similarly we found that addition of IL-6 increased the IL-4-dependent IgE production by human B cells "in vitro".

IL-5, IL-7 and IL-9 had no direct effect on proliferation or IgE synthesis. This was in contrast with other investigations in which IL-7 and IL-9 were reported to enhanced IgE production by human B cells. In these studies PBMC were used, thus the potentiating effect of these cytokines might be indirect, via other cell types (150) and (148). IL-5 can provide the trigger for isotype switching to IgG1 and IgE, by inducing the accumulation of productive γ1 and ε transcripts in PBMCs (147). The unresponsiveness of the human B cells to IL-5 could not be explained in our study.

Despite the general notion that IL-10 is an immunosuppressive cytokine, in our study IL-10 increased B-cell proliferation and IgE secretion. It has to be noted though that IL-10 (together with IL-6) have been suggested to be involved in B-cell lymphoproliferation acting "in vivo" in a paracrine fashion via T cells (335). IL-10, (as a type 1 or type 2 cytokine), has brought about one of the biggest contradictions concerning its effects in IgE switching. Initially it was strongly believed that IL-10 was immunosuppressive for IgE switching (153) and (317). More recent studies using peripheral B cells from patients with certain forms of leukaemia, such as lymphocytic leukaemia (CLL), hairy-cell leukaemia (HCL) and HCL variant (HCLV), have shown that IL-10 can upregulate the IgE switching process "*in vitro*", even in the absence of exogenous IL-4 (336). It is quite likely that IL-10 has a dual effect on B cells regarding IgE production. IL-10 expands B cells that have already switched to IgE by the action of

IL-4 and anti-CD40, but at the same time it might have a direct effect on these cells as the levels of IgE measured in the cultures were very high.

Finally, the suppressive effect of TGF-β on the IL-4-dependent IgE switching was probably due to the specificity of the latter to switch B cells to IgA. This property of TGF-β has been widely established (337). Indeed, the suppression of IL-4-dependent IgE secretion by the B cells was almost 100% at the higher concentrations of TGF-β as well as B-cell proliferation. From this it was concluded that the suppression of IgE synthesis by TGF-β was due to the inhibition of B-cell proliferation and stimulation of IgA production.

The proliferative effect of IL-2 on B cells was in concordance with previous findings. Gearing *et al.*, (1985) had shown that human B cells could proliferate with IL-2 alone, even in the absence of anti-CD40 monoclonal antibody (338). The existence of IL-2R on the surface of the human B cells, was demonstrated by Zubler *et al.*, (144). The decrease of IgE secretion by the human B cells due to the presence of IL-2 agrees with most of the literature. Miyajima *et al.*, (1991), reported that IL-2 could inhibit IL-4 induced IgE secretion of LPS-activated murine B cells (145). IL-2 appears to inhibit IL-2 secretion in a different manner compared to IFN-γ. IFN-γ was only inhibitory when present early during culture (339). In contrast, IL-2 inhibited most when added on day 4 or 5, indicating that IFN-γ interferes with an early step, and IL-2 with a late step in this IL-4-dependent B cell function.

IL-12 decreased IgE secretion by the human B cells in a dose-dependent fashion. The suppressive nature of IL-12 is widely established (156) and (157), and is believed to be indirect as it induces IFN-γ production by human CD4+ T cells. In our experiments.

even in the highly purified B cell cultures (positively selected with CD19+ dynabeads). IL-12 had a downregulatory effect on the IL-4 induced IgE secretion process. This suggests the possibility that it induces IFN-γ production by B cells. The IL-12 signal is transduced through the IL-12R and STAT 4 (340). B-cell proliferation was not affected by the presence of IL-12 in the cultures (1100 ± SEM).

Recently, the effects of IL-12 on IgE switching have been related to IL-18. Extensive studies have shown a homology between the IL-12 and the IL-18 receptors in humans indicating similar signalling pathways (341). Both receptors have been detected on naive human B cells and a synergistic effect between the two cytokines has been demonstrated regarding the induction of IFN-γ secretion by B cells (342), (343), (344) and (157).

IL-18 upregulated IL-4-dependent IgE synthesis while simultaneous addition of IL-12 and IL-18 brought IgE production down to control levels.

Finally, the purification method used to isolate the tonsillar B cells, did not bias the effect of the exogenous cytokines on the IL-4-dependent B cell proliferation and IgE synthesis. The overall lower levels of IgE produced by the positively selected B cells underlines the sensitivity of the process, while the higher proliferation levels were probably due to the stimulation by the CD19+ dynabeads during the purification method. It is likely that the CD19 dynabeads are toxic to B cells, possibly inhibiting all types of immunoglobulin class switching. For this reason, T-cell resetting was extensively preferred over positive selection for B-cell purification.

CHAPTER 5: CYTOKINES AND THEIR MODES OF ACTION IN IgE REGULATION "*IN VITRO*".

5.1 INTRODUCTION.

In this chapter, we assessed the possible mode of action by which cytokines were found to influence IgE secretion by human B cells. As described in chapter 4, such cytokines include: IL-6 and IL-10, found to potentiate IL-4 secretion and IL-2, IL-12, IFN-γ and TGF-β, which inhibited this process. In the case of IL-6 and IL-10, it would be interesting to see whether the increase in IgE secretion was a direct effect of these cytokines on the switching of human B cells to IgE or through the expression of committed precursors. IL-6 is a multifunctional cytokine important for the differentiation of human B cells into Ig secreting cells (345). IL-6 has been shown to expand B cell precursors and caused B cell proliferation both *in vivo* and *in vitro* (346). The synergistic effect of IL-6 with IL-4 in upregulating IgE switching has been shown with both purified human B cells (347), and in mixed PBMC cultures (127).

IL-10, a lymphokine produced by various cell types such as T cells, mast cells and macrophages, has been widely characterised as a suppressive agent in inflammatory responses (348). It has been also classified as a B cell growth factor (among IL-2 and IL-4) (327), causing proliferation and plasma cell development in germinal centres (349). The augmenting effect of B cell proliferation by IL-10 was also shown by Nagumo and Agematsu (1998), when B cells were cultured in the presence of IL-4 and CD27 ligand (CD70) (350).

The way in which cytokines inhibit Ig production may involve a number of different mechanisms, such as the stimulation of B cells to produce a different Ig subclass seen in the case of IFN-γ and TGF-β triggering the secretion of IgG1 and IgA respectively. The

genetic mechanisms underlying these processes are mutually exclusive. thus switching one immunoglobulin isotype is irreversible and excludes the secretion of any other Ig subclass.

The importance of IL-4 in IgE production has been shown in chapters 3 and 4. This lead us to study the regulation of the IL-4 receptor on the human B cells. exerted by cytokines. There are two types of IL-4R: Class I (IL-4Rα/IL-2Rγ) expressed on B and T cells. and class II (IL-4Rα/L-13Rα) expressed on endothelial cells (351). (352). The shared hybrid component of the IL-4 and IL-13 receptors shares signalling pathways thus explaining the common biologic features between the two cytokines including IgE isotype switching, CD23 induction and eosinophilic activity (353). (354). IL-4Rα is a critical component for the binding of IL-4 and signal transduction of the latter cytokine (355). (356). (357). (358) and (359). Binding of IL-4 to its receptor induces phosphorylation and DNA binding activation of the signal transducer and activator of transcription (STAT) 6 protein (360). When STAT 6 was deleted in mice, inhibition of Th2 function and IgE synthesis was anticipated and confirmed (361). Subsequently, the IL-4Rα chain has been reported to be linked with atopy (inherited predisposition to make IgE to common inhaled antigens) (362) and (363). Consequently, regulation of the IL-4R expression may be a control to the study of IgE switching. It has been also shown that B cells activated by IL-4 increase the expression of IL-4R on their surface (352). By contrast, IFN-γ was shown to downregulate the expression of the IL-4R on B cells both indirectly (activation of STAT 1) and by direct attenuation of the IL-4-induced STAT 6 activity (352). In this study, we assessed the effect of IL-2, IL-12, IFN-γ and TGF-β on the expression of the IL-4R on human tonsillar B cells.

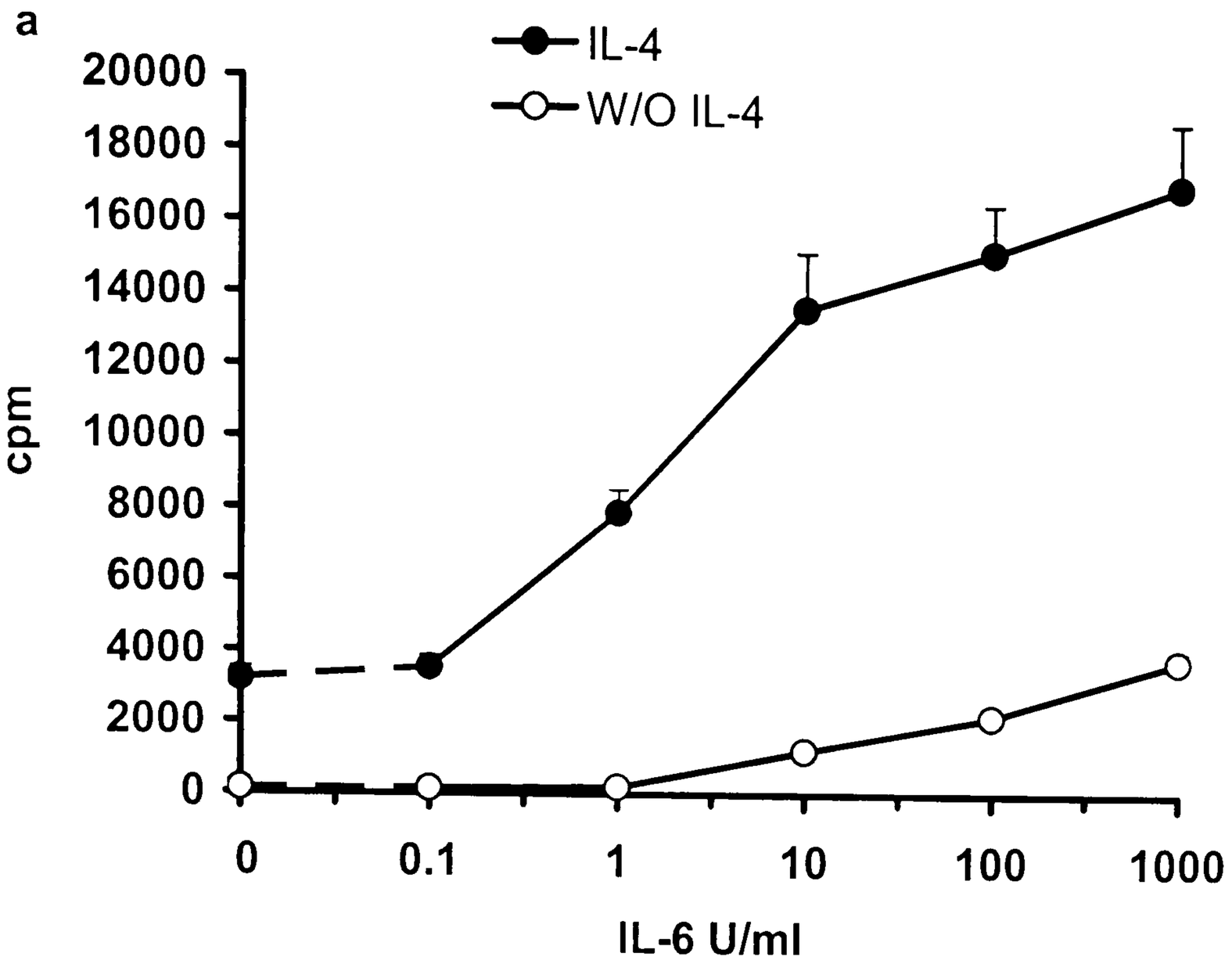
Finally, another possible way by which cytokines can regulate an Ig switching is by inducing the secretion of other cytokines, that can in turn inhibit the production of the primary immunoglobulin. The ability of IL-12, produced by dendritic cells and macrophages, to promote a type 1 immune response "in vivo" has been established (364). It has become apparent that B cells participating in TD responses can themselves manifest polarisation along Th1 or Th2 pathway very soon after antigen challenge with the direction taken depending on the nature of the antigen and the dominating cytokine profile at each time (365). In this study, we investigated the downregulatory effects of IL-12 on IgE secretion on the basis of IFN-γ production by the human B cells.

5.2 RESULTS.

5.2.1 Analysing the effects of IL-6 and IL-10 on B cell proliferation and IgE secretion.

Tonsillar B cells were isolated by T-cell rosetting and cultured with anti-CD40 (0.5 μg/ml), and IL-6 or IL-10 (0-1000 U/ml) in the presence or absence of IL-4 (20 ng/ml). Following a four day culture period, the cell cultures were pulsed with ^{3}H-Thymidine and counted in a β-counter were related to assess proliferation. The effect of IL-6 and IL-10 on the B-cell proliferation levels was similar. As previously shown (chapter 4), IL-6 and IL-10 potentiated B cell proliferation in a dose-dependent fashion in the presence of IL-4. Similar results were observed in this case, with IL-10 being more potent compared to IL-6, as the former gave maximum proliferation levels of 27000 cpm as opposed to 18000 cpm obtained with IL-6 (figures 5.1 + 5.2). In the absence of IL-4, both IL-6 and IL-10 had a five-fold less immunoproliferative effect on the human B cells.

After a ten day culture period B-cell supernatants were collected and the IgE levels were measured by a direct IgE sandwich ELISA. Again as previously shown in chapter 4, the synergistic effect of IL-6 and IL-10 with IL-4 in our IgE secretion system was proven. IgE levels increased with increasing amounts of IL-6 and IL-10 with the latter being more potent giving IgE levels that reached 2000 ng/ml. Neither of the two cytokines was able to switch B cells to IgE secretion in the absence of IL-4 (figures 5.1 and 5.2).



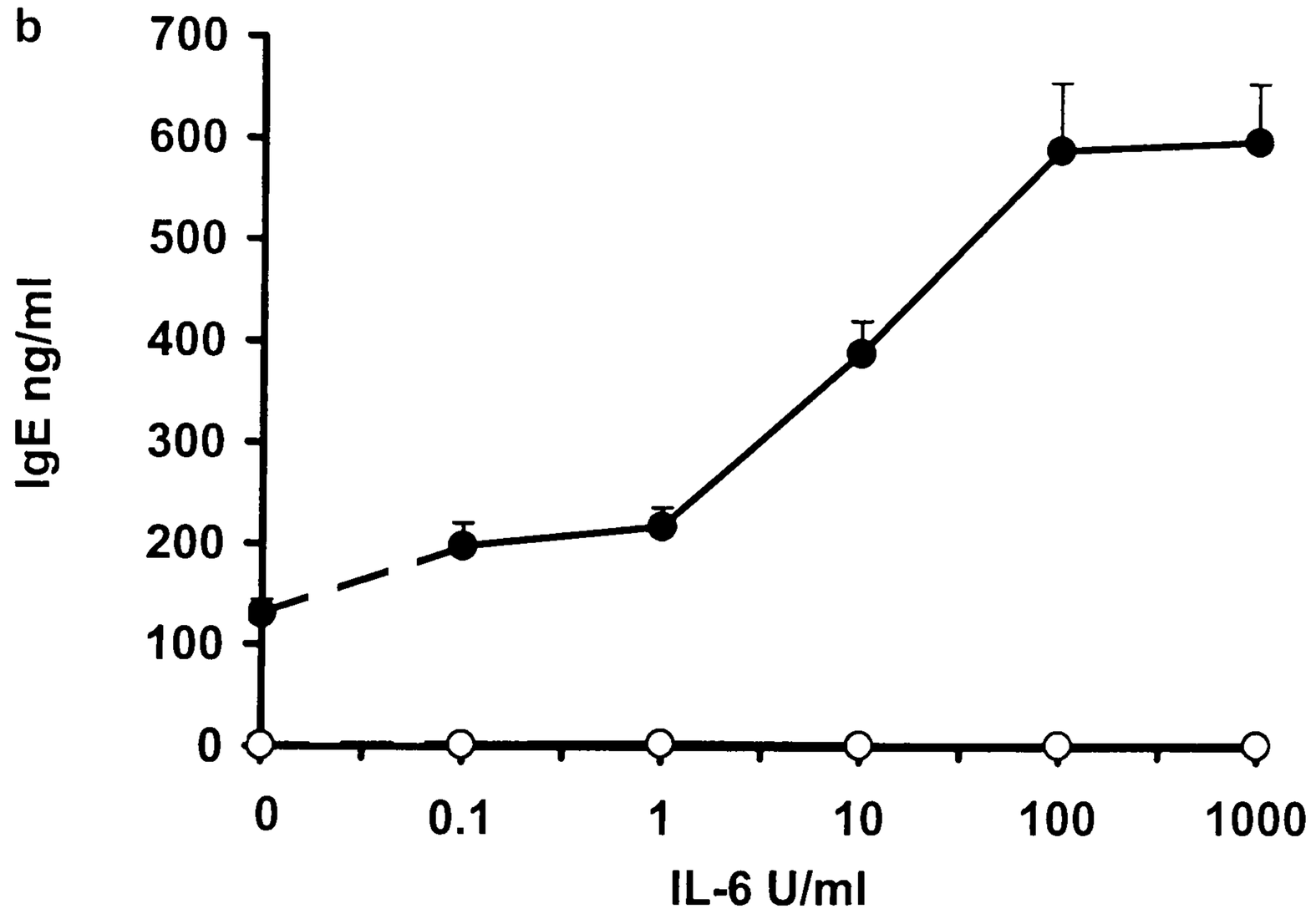
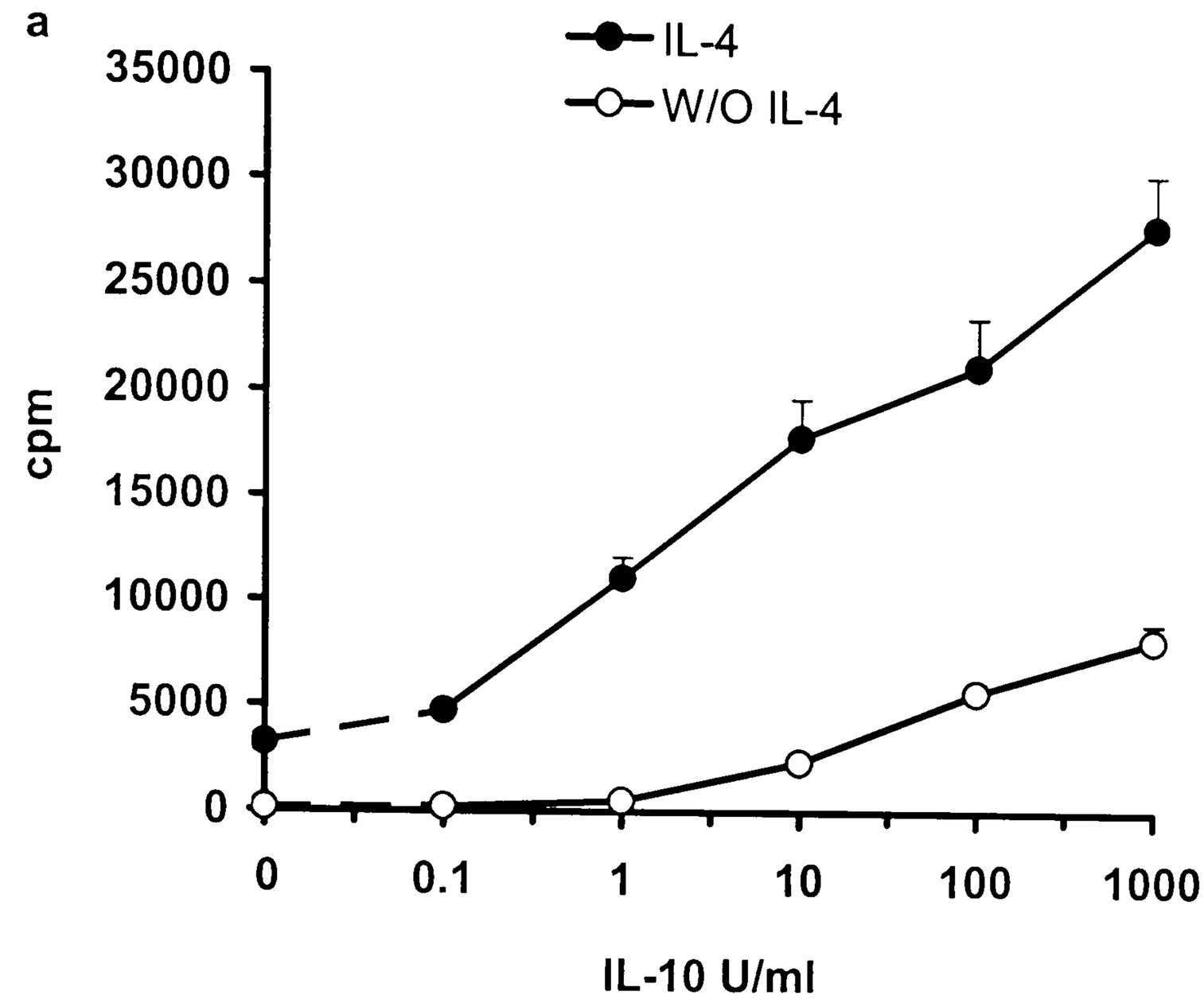


Fig. 5.1: Effect of IL-6 on B cell-proliferation and IgE secretion in the presence or absence of IL-4. Tonsillar B cells (from one donor), were purified by T-cell rosetting and cultured with anti-CD40 (0.5 μg/ml) and incremental amounts of exogenous IL-6, in the presence (closed circles) or absence (open circles) of IL-4 (20 ng/ml). B-cell proliferation was measured by the incorporation of ^{3}H-Thymidine after 4 days of culture (a) while the IgE levels were measured ELISA after 10 days of culture (b). These results are representative of three donors and the standard deviations have been calculated (triplicates).



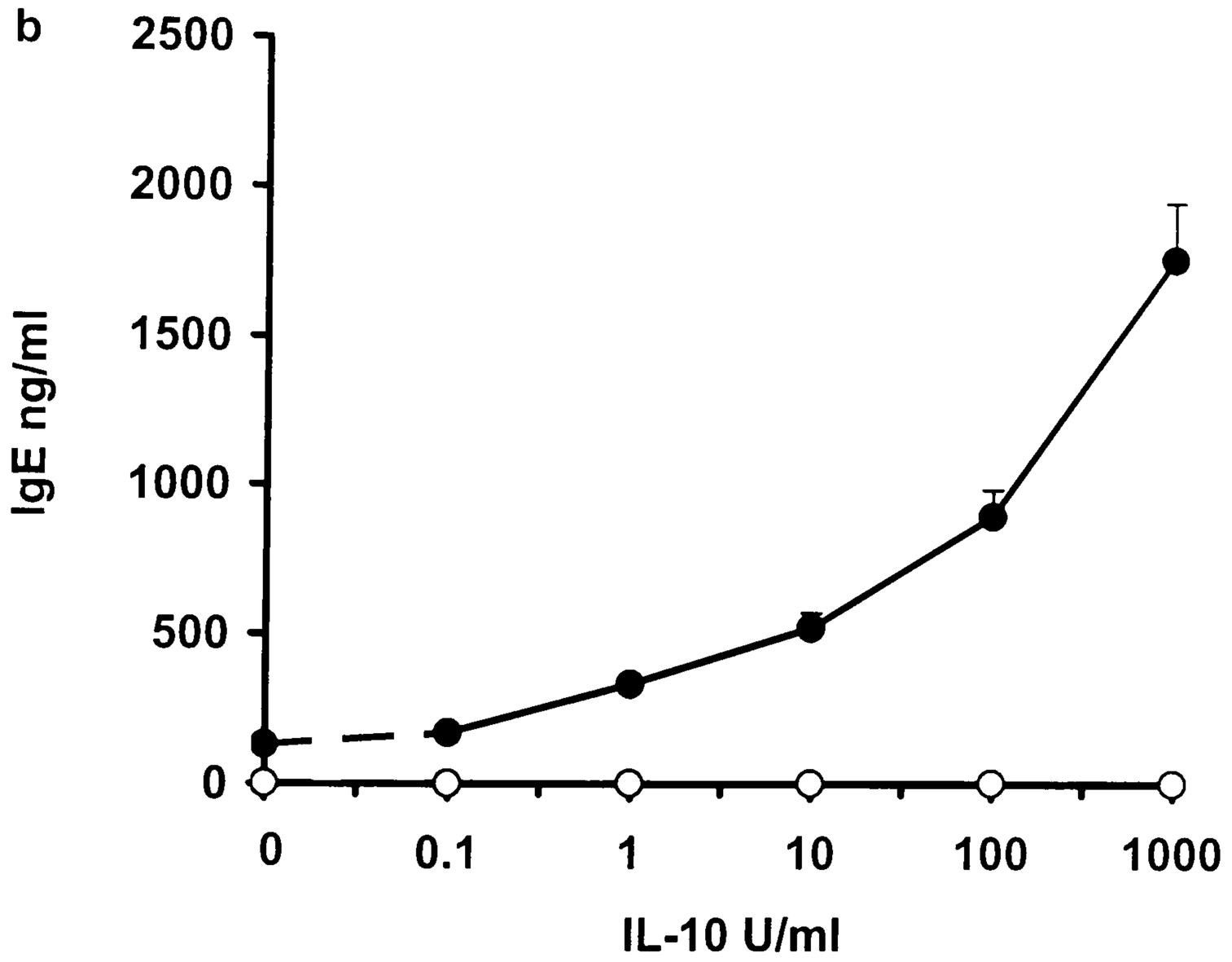


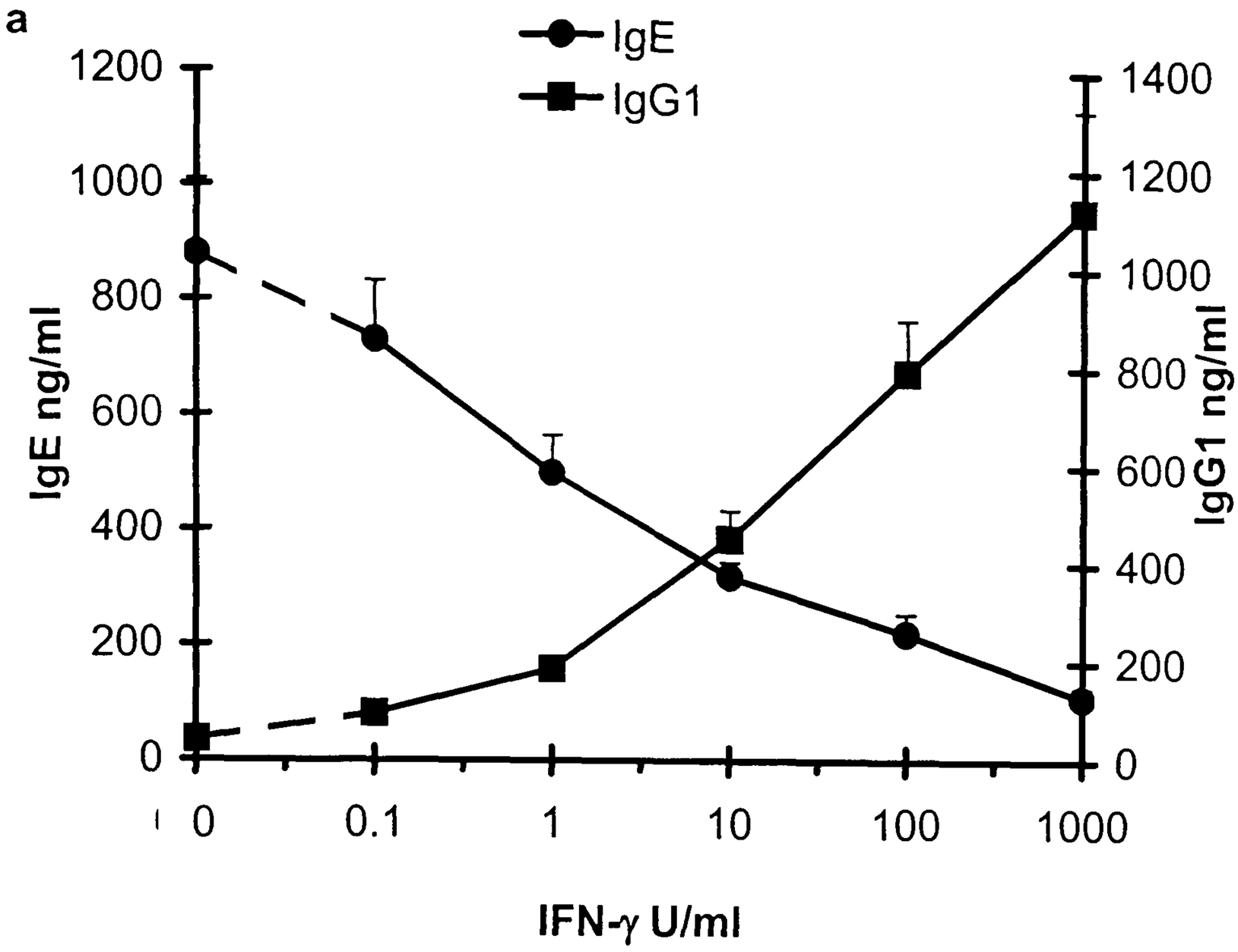
Fig. 5.2: Effect of IL-10 on B cell-proliferation and IgE secretion in the presence or absence of IL-4. Tonsillar B cells (from one donor), were purified by T-cell rosetting and cultured with anti-CD40 (0.5 μg/ml) and incremental amounts of exogenous IL-10, in the presence (closed circles) or absence (open circles) of IL-4 (20 ng/ml). B-cell proliferation was measured by the incorporation of ^{3}H-Thymidine after 4 days of culture (a), while the IgE levels were measured by ELISA after 10 days of culture (b). Results are representative of three donors and the standard deviations have been calculated (triplicates).

5.2.2 IFN-γ and IgG1 secretion.

Tonsillar B cells were isolated by T-cell rosetting and cultured with anti-CD40 (0.5 μg/ml), titrated amounts of IFN-γ (0-1000 U/ml) in the presence or absence of IL-4 (20 ng/ml). Following a 10 day culture, B-cell supernatants were collected and the IgG1 and IgE levels measured by direct sandwich IgG1 and IgE ELISAs respectively. Figure 5.3a shows the ability of IFN-γ to upregulate the IgG1 levels and downregulate the IgE levels secreted by the human B cells "in vitro", under the presence of IL-4. A five-fold decrease in the levels of IgE was observed at 1000 U/ml of IFN-γ while the highest levels of IgG1 obtained were 1150 ng/ml. The IgG1 switching ability of IFN-γ was confirmed by the dose-dependent increase of the antibody in the absence of IL-4 (figure 5.3b). IL-4, at 20 ng/ml, did not inhibit IgG1 production but IFN-γ inhibited IgE at 1 ng/ml.

5.2.3 TGF-β and IgA secretion.

Tonsillar B cells were isolated by T-cell rosetting and cultured with anti-CD40 (0.5 μg/ml), titrating amounts of TGF-β (0-1000 U/ml) and in the presence or absence of IL-4 (20 ng/ml). Following a ten day culture, B-cell supernatants were collected and the IgA and IgE levels measured by an IgA ELISA kit and a direct sandwich IgE ELISA respectively. Figure 5.4a shows the ability of TGF-β to increase the IgA levels and suppress IgE secreted by the human B cells "in vitro", in the presence of IL-4. A complete suppression of IgE secretion was observed at 1000 U/ml of TGF-β while the highest levels of IgA obtained were 860 ng/ml. The IgA switching ability of TGF-β was



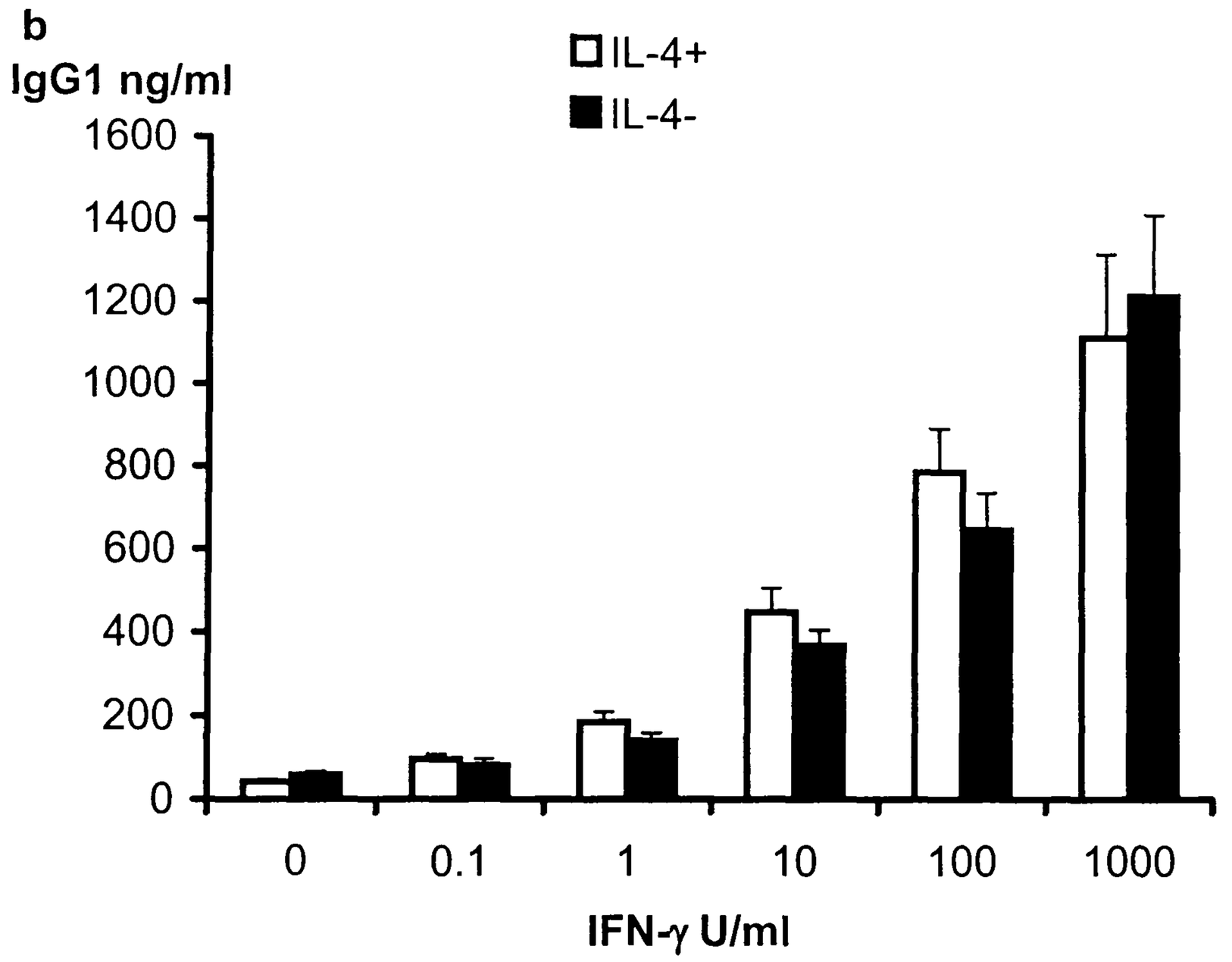
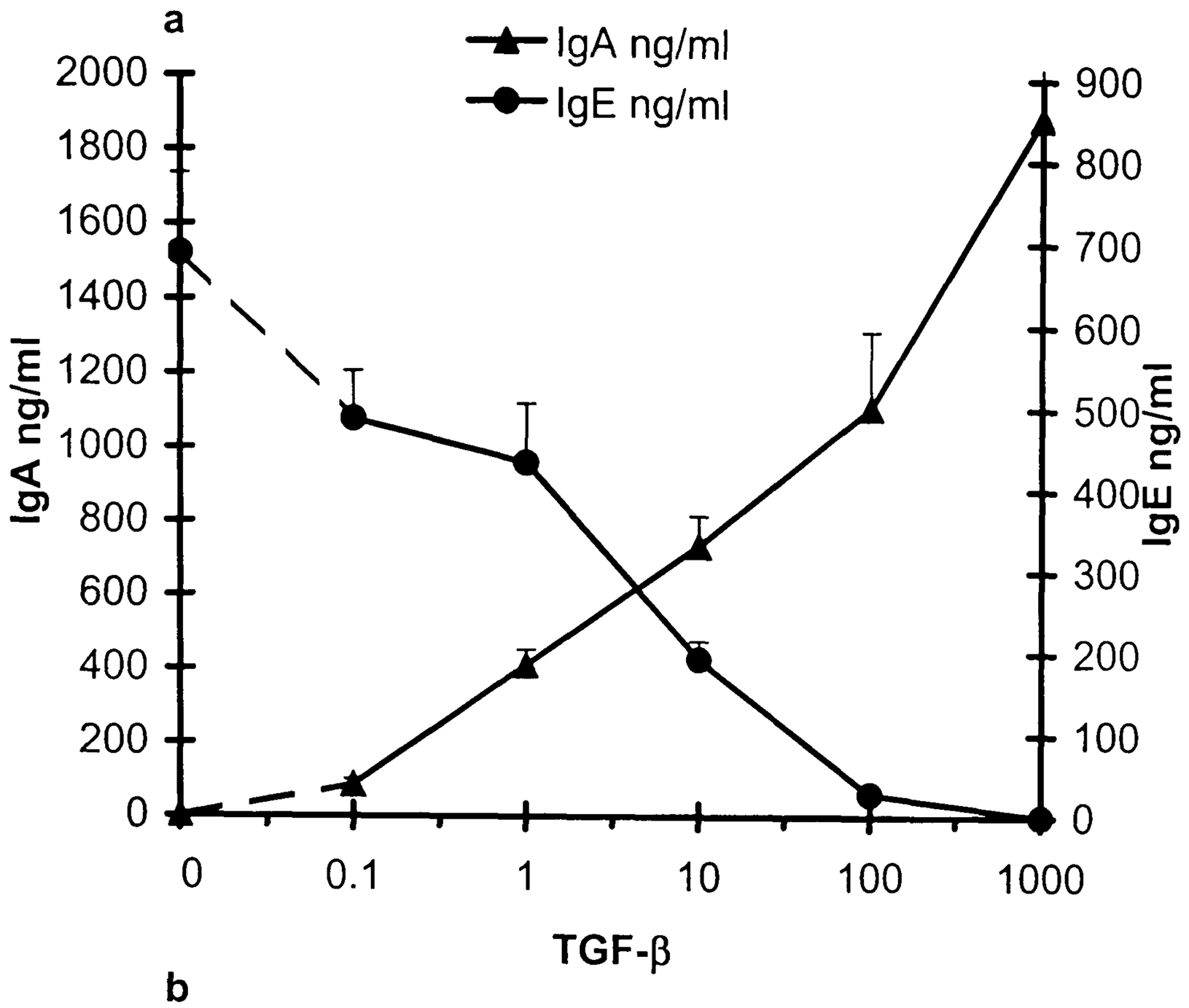


Fig. 5.3: Effect of IFN-γ on IgG1 and IgE secretion. Tonsillar B cells isolated by T-cell rosetting were cultured with IL-4 (20 ng/ml), anti-CD40 (0.5 μg/ml) and increasing amounts of IFN-γ (a). B cells were also cultured with IFN-g but in the absence of IL-4 (b). After 10 days of culture the supernatants were collected and the IgG1 and IgE levels were measured by ELISA. The results are the average of three donors and the standard errors have been calculated.



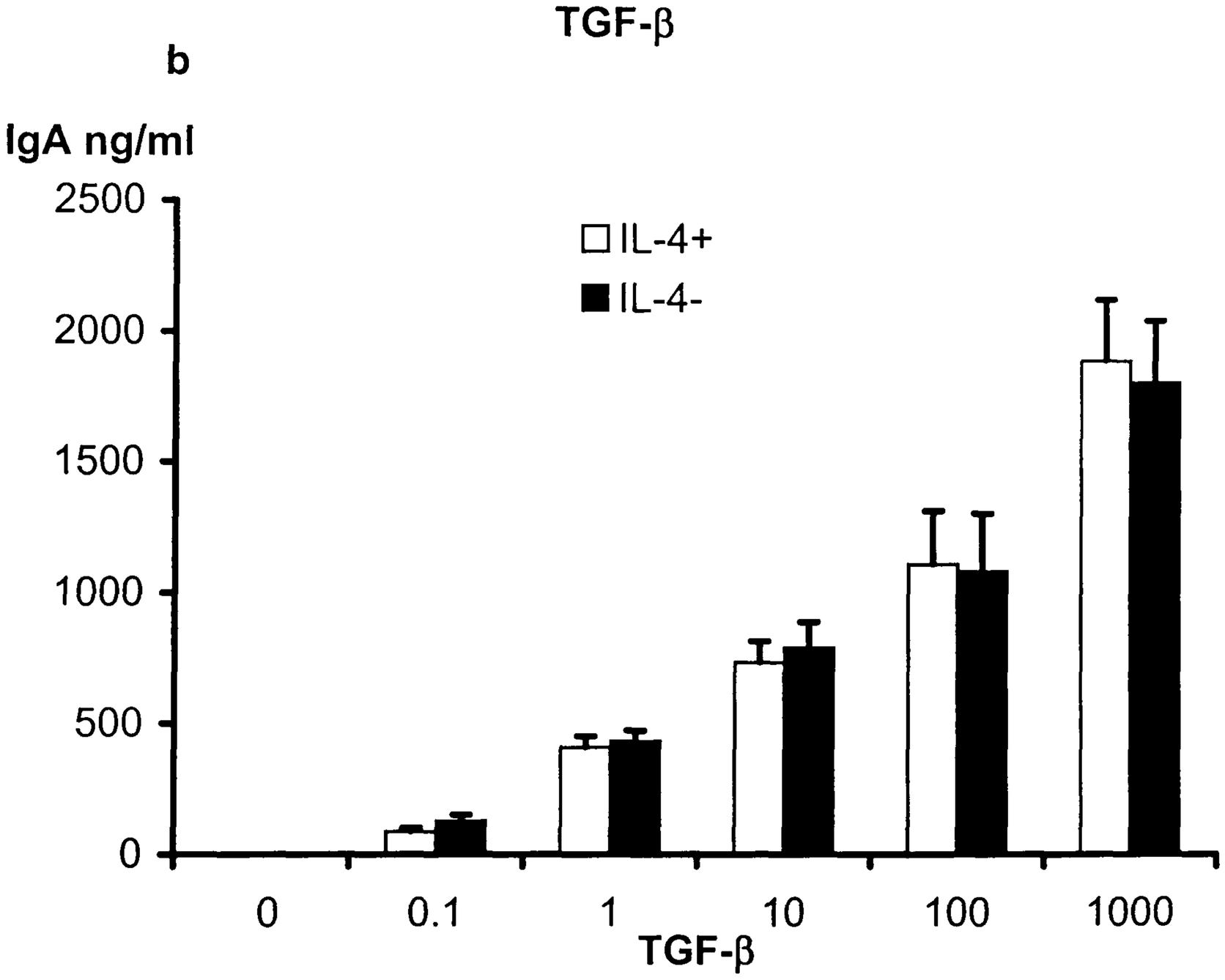


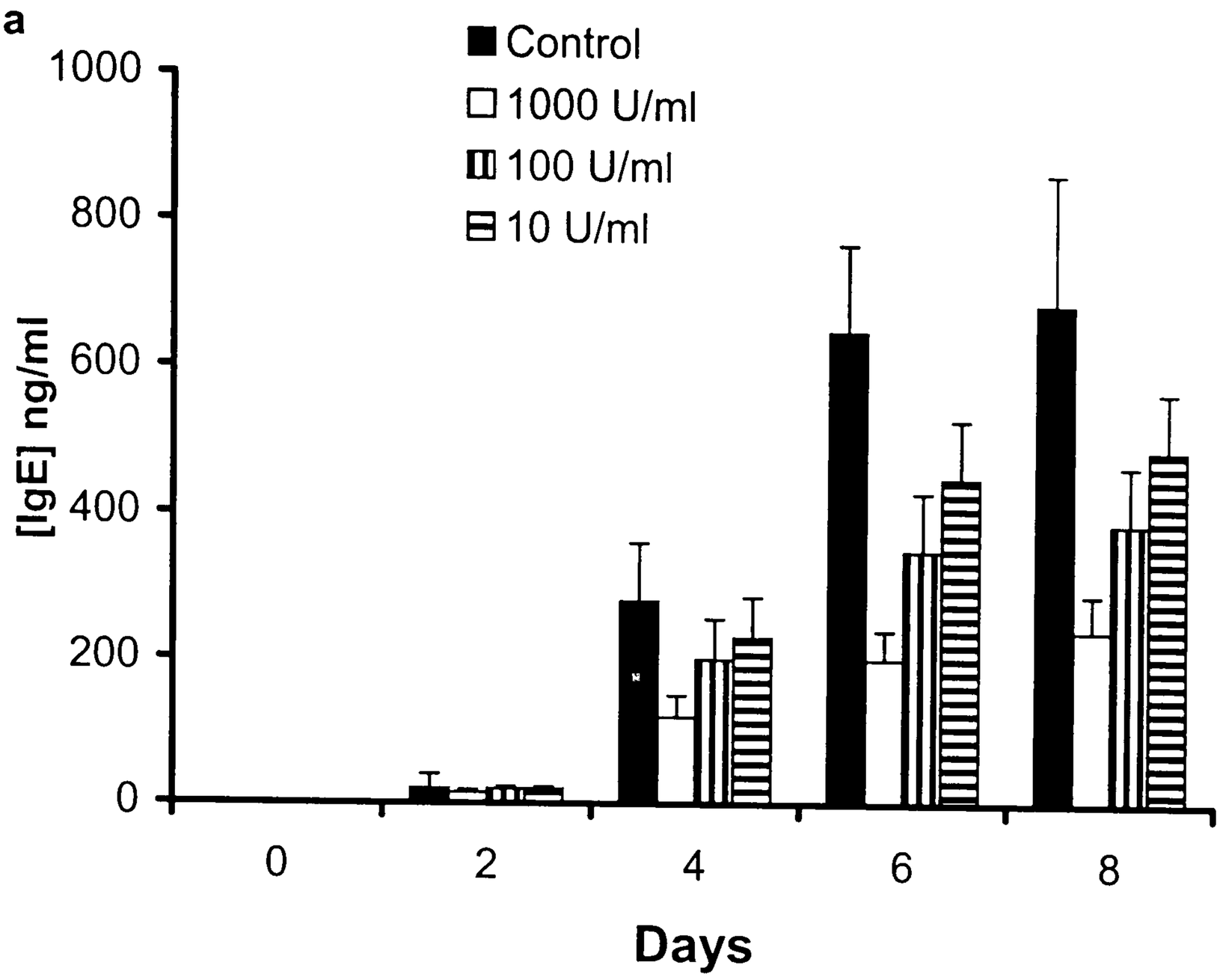
Fig. 5.4: Effect of TGF-β on IgA and IgE secretion. Tonsillar B cells isolated by T-cell rosetting were cultured with IL-4 (20 ng/ml), anti-CD40 (0.5 μg/ml) and incraesing amounts of TGF-β (a). B cells were also cultured with TGF-β but in the absence of IL-4 (b). After 10 days of culture the supernatants were collected and the IgA and IgE levels were measured by ELISA. The results are the average of three donors and the standard errors have been calculated.

confirmed by the dose-dependent increase of the antibody in the absence of IL-4 (figure 5.3b). IL-4 was unable to inhibit IgA secretion at 20 ng/ml.

5.2.4 IL-12-dependent IFN-γ secretion by human B cells.

Tonsillar B cells were isolated by T-cell rosetting and cultured with IL-4 (20 ng/ml), anti-CD40 (0.5 μg/ml) and titrated amounts of IL-12 (0-1000 U/ml). The B cell supernatants were collected at days: 0, 2, 4, 6 and 8, and the IgE and IFN-γ levels were assessed by direct IgE and IFN-γ ELISAs. Figure 5.5a shows a downregulation of the IL-4-dependent IgE secretion with increasing amounts of IL-12 (also shown in chapter 4). This inhibition became more apparent after day 6, and was greater at 1000 U/ml of IL-12.

Figure 5.5b depicts increasing IFN-γ levels with increasing concentration of IL-12 on day 6 and onwards. The maximum levels of IFN-γ secreted by the B cells, were observed after 8 days of culture with 1000 U/ml IL-12, reaching the mark of 20 ng/ml. Any IFN-γ picked up prior to day 6 or under control conditions (with IL-4 and anti-CD40 solely), were considered as background levels. The levels of IFN-γ detected in the B-cell cultures after day 6 were too high to be secreted by the contaminated T cells.



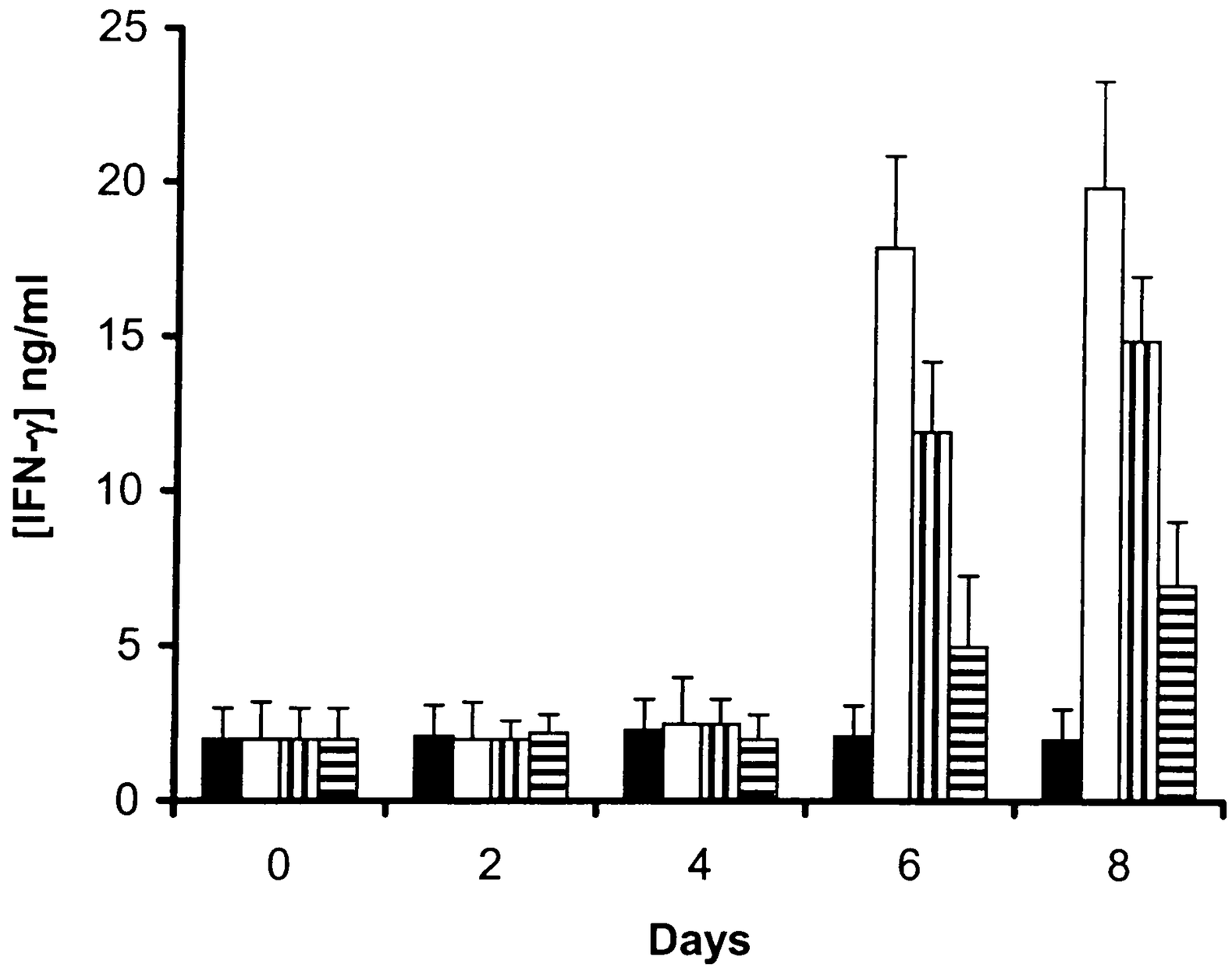


Fig. 5.5: Effect of IL-12 on IgE secretion and IFN-γ production by human B cells. Tonsillar B cells were isolated by T-cell rosetting and cultured with IL-4 (20 ng/ml) and anti-CD40 (0.5 μg/ml), and increasing amounts of IL-12. At two day intervals the supernatants were collected and the IgE levels (a) and the IFN-γ levels (b) were measured by ELISA. These results are representative of three donors and the standard deviations have been calculated (triplicates).

5.2.5 Cytokine regulation of IL-4 receptor expression on human B cells.

Cytokines that were found to have an effect on IL-4-dependent IgE secretion "*in vitro*" (chapter 4) were tested for their ability to regulate the expression of the IL-4R on B cells. Tonsillar B cells were isolated by T-cell rosetting and cultured with IL-4 (20 ng/ml) and anti-CD40 (0.5 μg/ml) as well as with one of the following cytokines: IL-6, IL-10, IL-2, IL-12, IFN-γ and TGF-β at 100 U/ml. At days: 0, 2, 4, 6 and 8 the cells were collected and put through an acid wash stage (HCl – pH 4.1) in order to strip any exogenous IL-4, added at day 0, that was still bound on the IL-4R. After the acid wash step, the cells were stained with CD19-FITC and biotinylated IL-4 followed by PE-conjugated streptavidin. The levels of the B cells expressing the IL-4R on their surface was assessed by flow-cytometry. As shown in figure 5.6 the levels of B cells expressing the IL-4R was very high under control conditions (IL-4 and anti-CD40 solely) (around 96% on average). This expression was time-independent within 8 days of the study. Similar levels of expression were observed when the B cells were cultured in the presence of IL-6, IL-10 (figure 5.7) and IL-2 (figure 5.8). The level of IL-4R expression did not change with time and was unaffected by these cytokines. IL-12 downregulated the IL-4R expression by 30% after 8 days of culture (figure 5.8), while IFN-γ and TGF-β brought about a 50% inhibition of the IL-4R expression by day 4 (figure 5.9). The effect of cytokines on the IL-4R expression on human B cells have been summarised in figure 5.10. Due to the high levels of the IL-4R observed on tonsillar B cells at day 0, PBMC derived B cells were also stained for the receptor. The levels of the IL-4R were 31.9% (figure 5.6).

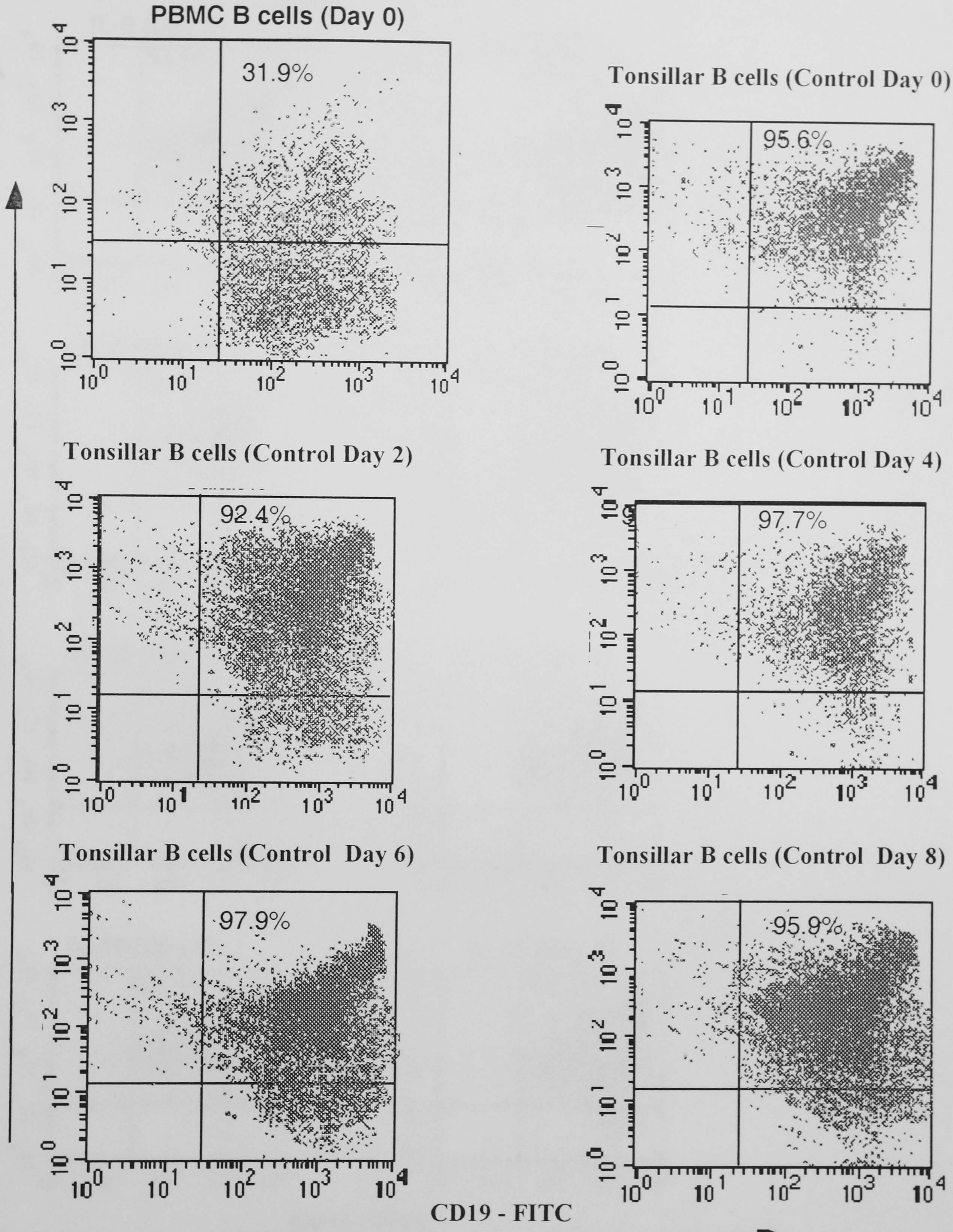


Fig. 5.6 : Effect of IL-4 and anti-CD40 (control conditions) in the expression of IL-4R. on human B cells. Tonsillar B cells were isolated by T-cell rosetting and cultured with IL-4 (20 ng/ml) and anti-CD40 (0.5 μg/ml). At days: 0, 2, 4, 6 and 8 the cells were collected, washed (acid wash) and surface stained. The B-cell purity was assessed using CD19-FITC monoclonal antibody while the expression of the IL-4R was measured indirectly by the binding of biotinylated IL-4 followed by the binding of PE-conjugated streptavidin. In the panels above, the B cells expressing IL-4R are shown (double positive). These results are representative of two experiments. PBMC derived B cells were isolated by T-cell rosetting and stained at day 0 for control purposes.

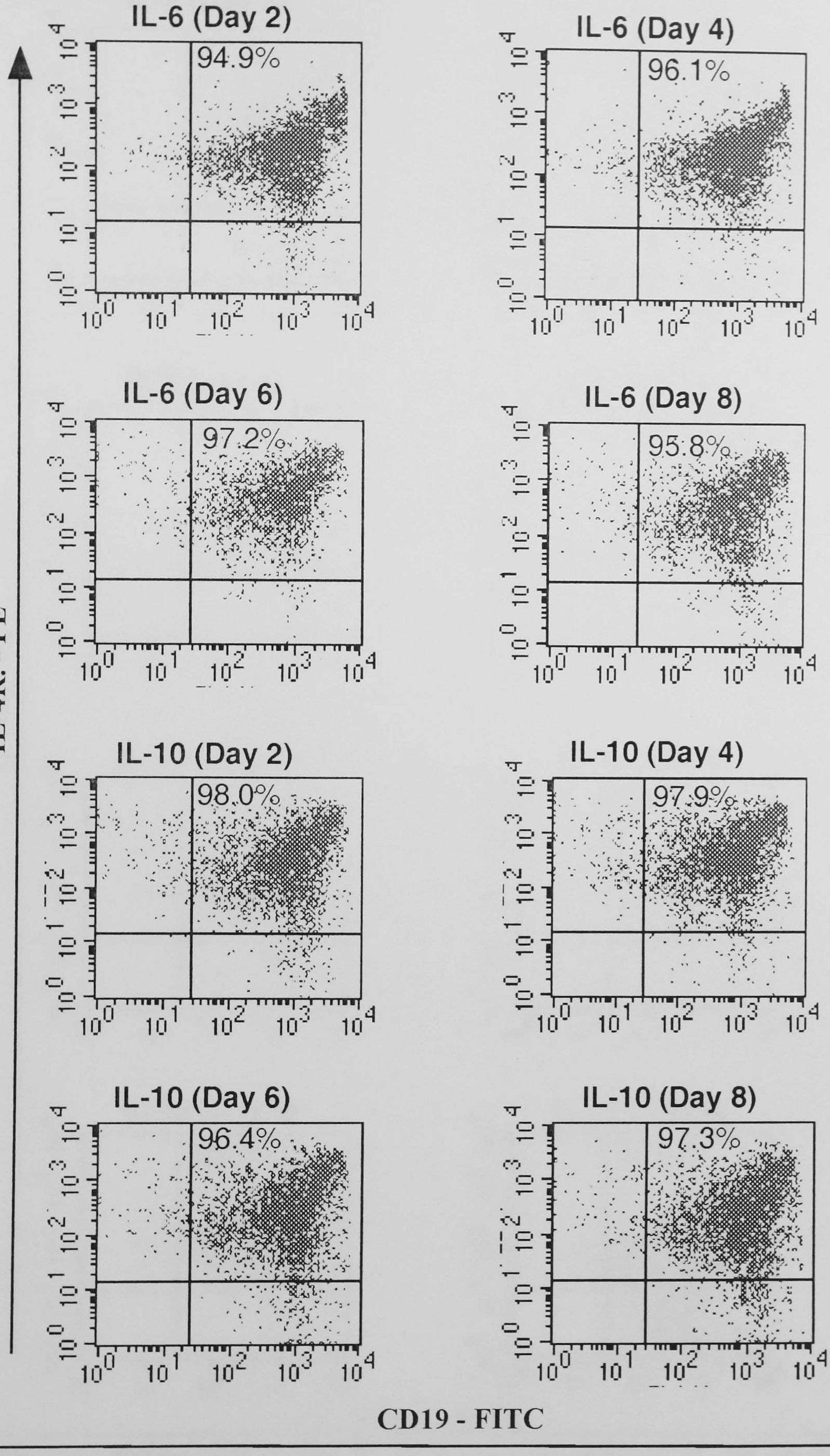


Fig. 5.7 : Effect of IL-6 and IL-10 in the expression of IL-4R on human B cells. Tonsillar B cells were isolated by T-cell rosetting and cultured with IL-4 (20 ng/ml), anti-CD40 (0.5 μg/ml) and 100 U/ml IL-6 or IL-10. At days: 2, 4, 6 and 8 the cells were collected, washed (acid wash) and surface stained. The B-cell purity was assessed usingt CD19-FITC monoclonal antibody while the expression of the IL-4R was measured indirectly by the binding of biotinilated IL-4 followed by the binding of PE-conjugated streptavidin. In the pannels above, the B cells expressing IL-4R are shown (double positive).

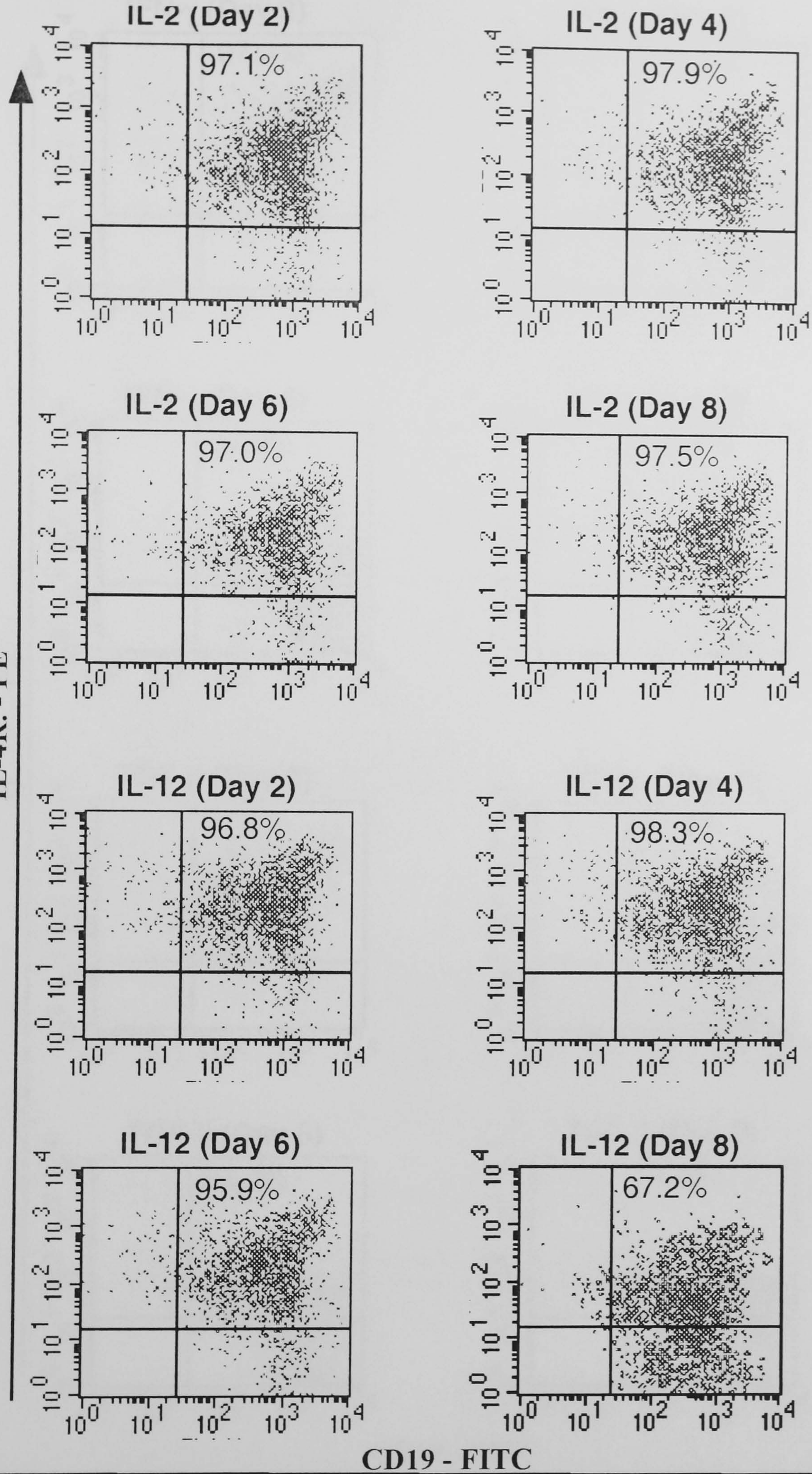


Fig. 5.8 : Effect of IL-2 and IL-12 in the expression of IL-4R. on human B cells. Tonsillar B cells were isolated by T-cell rosetting and cultured with IL-4 (20 ng/ml), anti-CD40 (0.5 μg/ml) and 100 U/ml IL-2 or IL-12. At days: 2, 4, 6 and 8 the cells were collected, washed (acid wash) and surface stained. The B-cell purity was assessed using CD19-FITC monoclonal antibody while the expression of the IL-4R was measured indirectly by the binding of biotinylated IL-4 followed by the binding of PE-conjugated streptavidin. In the panels above, the B cells expressing IL-4R are shown (double positive).

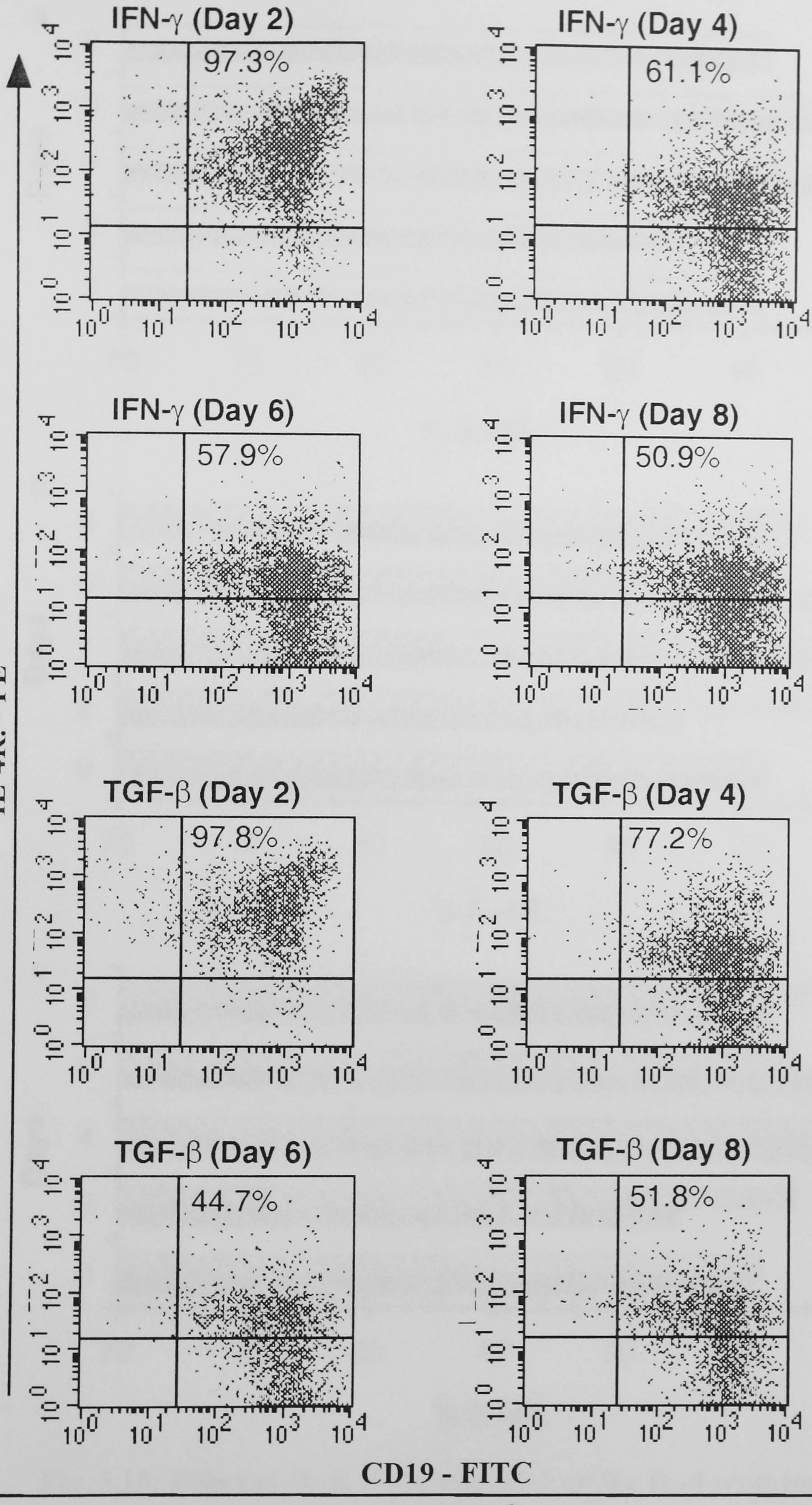


Fig. 5.9 : Effect of IFN-γ and TGF-β in the expression of IL-4R. on human B cells. Tonsillar B cells were isolated by T-cell rosetting and cultured with IL-4 (20 ng/ml), anti-CD40 (0.5 μg/ml) and 100 U/ml IFN-γ or TGF-β . At days: 2, 4, 6 and 8 the cells were collected, washed (acid wash) and surface stained. The B-cell purity was assessed using CD19-FITC monoclonal antibody while the expression of the IL-4R was measured indirectly by the binding of biotinylated IL-4 followed by the binding of PE-conjugated streptavidin. In the pan els above, the B cells expressing IL-4R are shown (double positive).

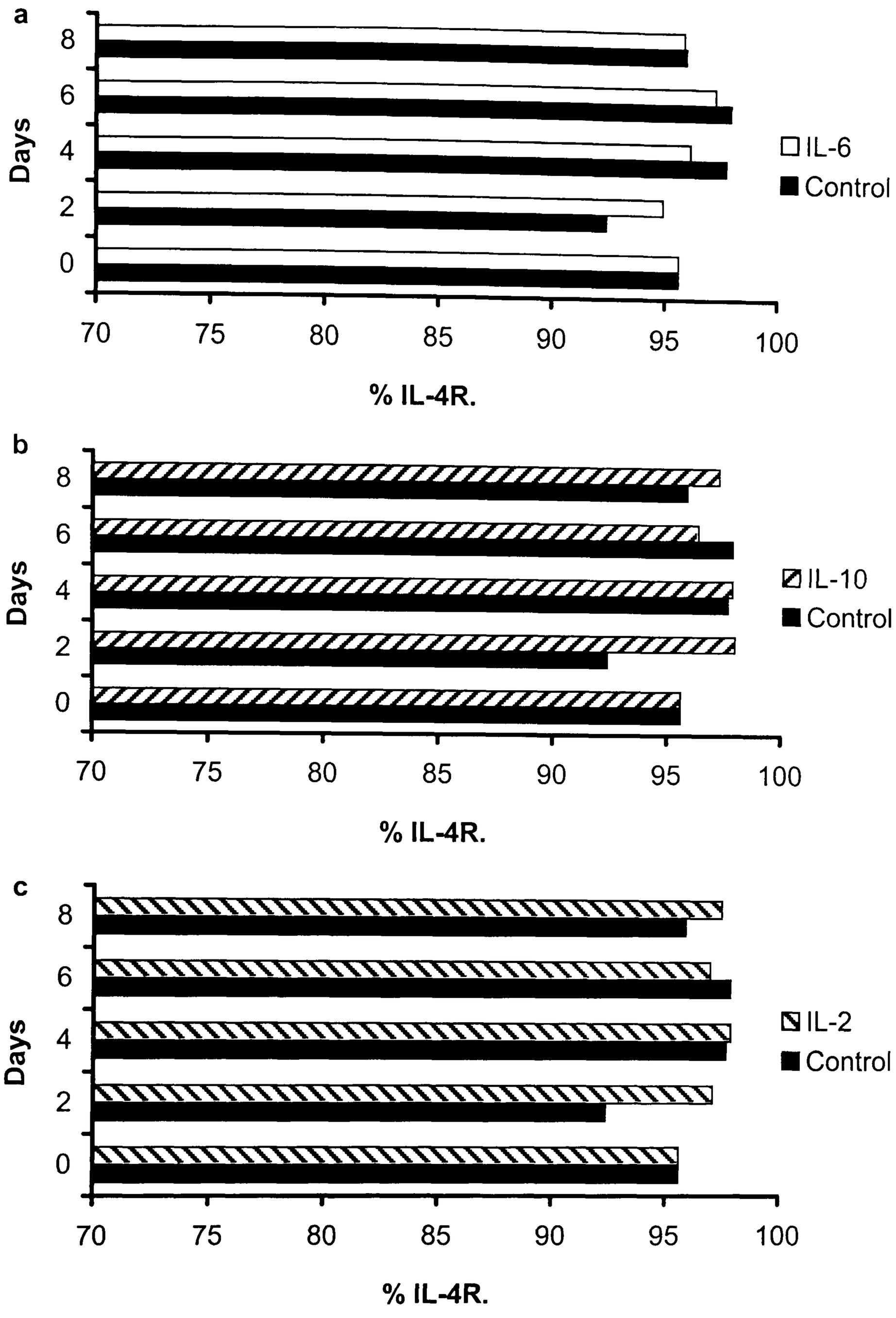


Fig. 5.10: Effect of IL-6, IL-10 and IL-2 on the IL-4 receptor expression on the surface of human B cells. Tonsillar B cells were isolated by T-cell rosetting and cultured with IL-4 (20 ng/ml), anti-CD40 (0.5 μg/ml) (control conditions) and one exogenous cytokine among IL-6 (a), IL-10 (b) and IL-2 (c) at a concentration of 100 U/ml. At days: 0, 2, 4, 6 and 8 the supernatants were collected and any bound IL-4 was stripped off by acid wash. B cells were double stained with CD19-PE and biotinylated IL-4 followed by streptavidin-PE. The % levels of the B cells expressing the IL-4R are depicted.

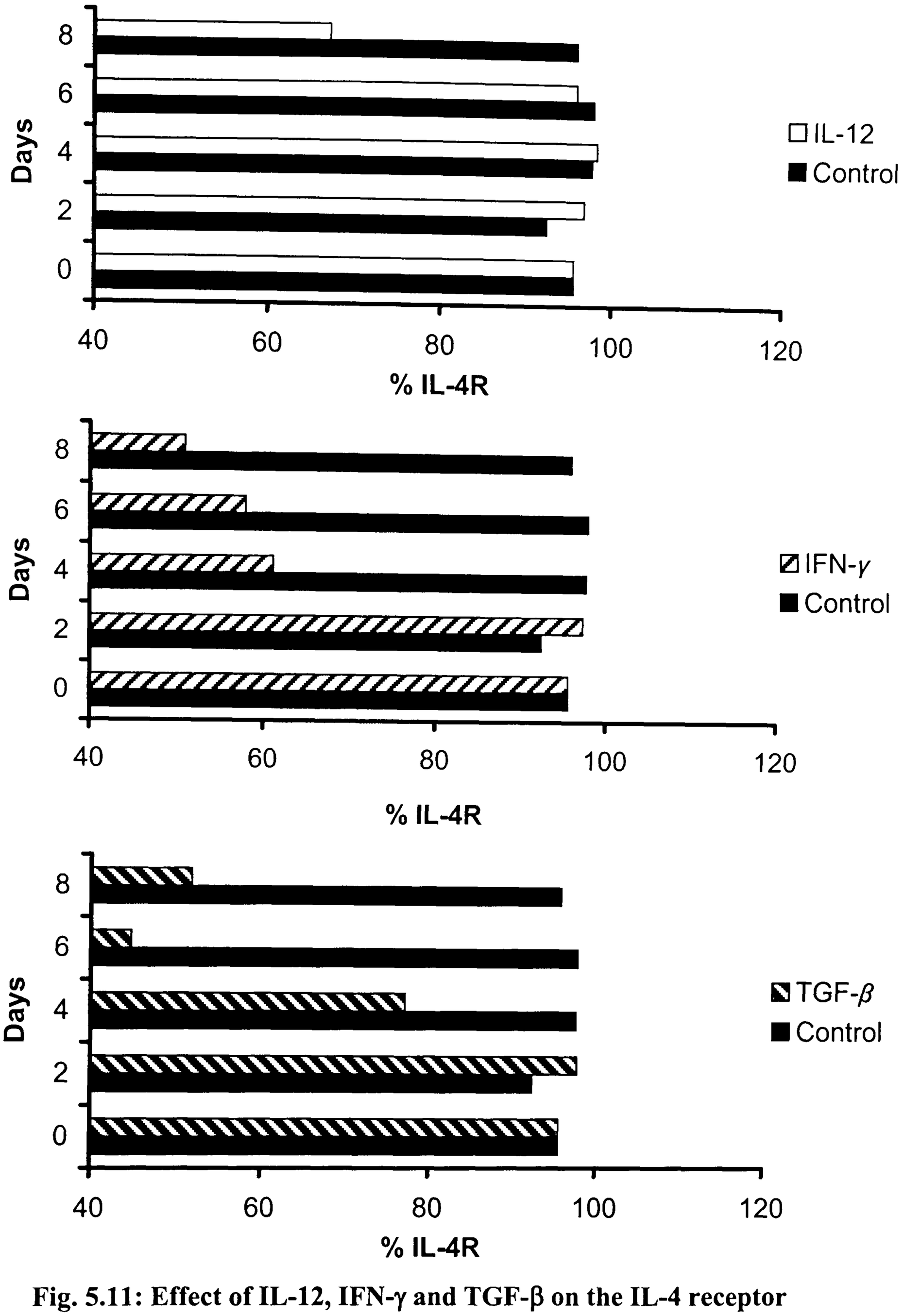


Fig. 5.11: Effect of IL-12, IFN-γ and TGF-β on the IL-4 receptor expression on the surface of human B cells. Tonsillar B cells were isolated by T-cell rosetting and cultured with IL-4 (20 ng/ml), anti-CD40 (0.5 μg/ml) (control conditions) and one exogenous cytokine among IL-12 (a), IFN-γ (b) and TGF-β (c) at a concentration of 100 U/ml. At days: 0, 2, 4, 6 and 8 the supernatants were collected and any bound IL-4 was stripped off by acid wash. B cells were double stained with CD19-PE and biotinylated IL-4 followed by streptavidin-PE. The % levels of the B cells expressing the IL-4R are depicted.

5.3 DISCUSSION.

In this study we investigated the possible modes of action of cytokines that were found to affect IgE secretion "in vitro". The importance of IL-6 in enhancing IgE production in the presence of IL-4. has been widely established (127). (347). IL-6 was found to be unable to switch B cells to IgE production in the absence of IL-4 and the B-cell proliferation levels were five-fold less. This was in concordance with the findings of Bjorck et al.. (1998). that showed the IgE switching ability of IL-6. and its strong B-cell proliferative activity, but only in the presence of IL-4 (346). Jeppson et al.. (1998). also demonstrated the capacity of IL-6 to enhance IgE class switching via an autocrine fashion. in B cells cultured with IL-4 and anti-CD40 (366). Thus, in our study. IL-6 synergises with IL-4 to promote B-cell proliferation while the IgE secretion process is entirely dependent on the presence of IL-4.

A similar case was that of IL-10. Interestingly. IL-10 was much more potent in inducing B cell proliferation and IgE production compared to IL-6. Again though, proliferation levels dropped five-fold in the absence of IL-4 and IL-10 alone was unable to drive IgE secretion by the human B cells in the absence of IL-4. The low proliferative effect of IL-10 in the absence of IL-4 was also demonstrated by Rousset et al.. (1992). although he showed that IL-4 could be substituted with an antigen (such as SAC particles). leading to the activation of the B cells via their BCR (327). The ability of IL-10 to enhance IgE secretion in the presence of IL-4 was. as with IL-6, due to its immunoproliferative activity on the B cells that were in turn stimulated by IL-4 to produce IgE. Jeannin *et al.*. (1998). demonstrated a dual role of IL-10 on IgE regulation. based on the time of its

addition to PBMC cultures (317). More specifically. IL-10 downregulated ε-transcript expression when added with IL-4 within the first three days of culture. Addition of IL-10 after day three, potentiated the IL-4-dependent secretion of IgE, possibly via clonal expansion of switched B cells. It is worth noting that the prior downregulation of IgE class switching was reversed by CD40 ligation which might explain the absence of IgE suppression in our system.

The downregulatory effects on IgE secretion by IFN-γ and TGF-β, were shown to be due to the preferential switching of these cytokines towards a different Ig subclass. More specifically, IFN-γ was found to stimulate IgG1 production independently of IL-4. The IgG1 switching ability of IFN-γ has been demonstrated (367).

A similar case was observed for TGF-β. The role of TGF-β in IgA switching is widely accepted (320), (321) and (69). "In vivo" IgA switching though has been shown to be more complicated as TGF-β can cause the secretion of low levels of IgA by murine lymphocytes, indicating that the latter cytokine is just one of the factors driving this switching process (322), (319). This differed from our study with human B cells "*in vitro*", and showed that even low levels of TGF-β (1 ng/ml) were able to drive B cells to IgA production. Hence the ability of IFN-γ and TGF-β to switch human B cells to IgG1 and IgA production explains their effect on downregulating IgE production.

The importance of IL-4 in IgE class switching, lead us to investigate the possible regulatory action of cytokines on the expression of the IL-4 receptor on human B cells. There are two types of IL-4R: Class I (IL-4Rα/IL-2Rγ) expressed on B and T cells, and class II (IL-4Rα/IL-13Rα) expressed on endothelial cells (351), (352). The high levels of IL-4R expression on tonsillar B cells on day 0 was quite surprising. The experiment

was repeated with three donors with the same results. Staining of PBMC derived B cells though showed a 31.9% expression of the IL-4R which was what would be expected according to the literature. Therefore the method of staining was considered reliable. Unfortunately, the low yield of B cells purified by PBMCs did not allow us to repeat the experiment with these cells. Addition of IL-4 and anti-CD40 sustained the high levels of the receptor on the tonsillar B cells up to day 8 of culture. Ikizawa et al., (1995) showed that IL-4 was able to upregulate the expression of its own receptor (IL-4R) on human lymphocytes, although the impact this might have on IgE class switching is still not clear (368). The ability of cytokines to regulate the expression of the IL-4R might give some indication of their effect on IgE regulation. In this study, IL-6 and IL-10 did not affect the levels of IL-4R on the B cells, but the results are hardly conclusive due to the pre-activation state of the tonsillar B cells. IFN-γ was able to downregulate the expression of the receptor from day 2 of culture, a finding that is in agreement with Young-So et al., (2000), demonstrating the dual action of IFN-γ which activates STAT 1 and inhibits STAT 6 (352). A novel finding was the similar suppression of IL-4R expression on the B cells by TGF-β. Hence both IFN-γ and TGF-β were thought to have at least two modes of inhibiting IgE secretion "in vitro".

Interestingly, IL-12 was found to downregulate the expression of the IL-4R on the B cells at day 8 of culture. This was thought to be due to the ability of the latter cytokine to induce IFN-γ production by human B cells, as shown in this study. Production of IFN-γ in the B-cell cultures was significant at higher concentrations of IL-12 by day 6 of culture. The indirect inhibitory action of IL-12 on the IgE class switching process is in concordance with the findings of Gagro and Gordon (1999), who established that IL-12

can direct B cell to shift towards a Th1 like phenotype via the endogenous production and autocrine action of IFN-γ in B cell cultures "in vitro" (369).

Cytokines that can affect IgE production "in vitro" may act via a number of different direct (like Ig class switching) and indirect pathways involving B cells as well as other cell populations. Further studies though are required in order to establish the interactions of these cytokines "in vivo" concerning IgE regulation.

CHAPTER 6: CD8+ SUPERNATANTS AND IgE PRODUCTION.

6.1 INTRODUCTION.

In order for the B cells to complete successfully their cell cycle they need two types of signals: The first type, called **competence signal,** can be provided solely by the Ag itself, especially if the latter is multivalent (TI Ag). This interaction can be highly important for the development of B cells during the Ag-dependant stage, as it aids the formation of the germinal centres in the lymph nodes and it has also been suggested to be involved in the induction of Ig class switching and phenotypic differentiation of the B cells (370). The second signal for B-cell maturation, called the progression signal, is provided by the cytokines, which according to their type, they influence the Ig-class isotype produced by the B cells. This is known as signal 1 in immunoglobulin class switching (129) and (134). For example, in work done by A. Cerruti *et al.*, (1998), using a homogeneous line of B cells (CL-01), they showed that IL-5 and TGF-β induce IgA class switching while high amounts of IL-4 (>100 U/ml) can cause switching to IgG4 and IgE production (166) and (371). Signal 2 is generated by the interaction between CD40 and CD40L, the latter being present on activated T-cells (129).

The importance of T cells in IgE secretion can be demonstrated by the fact that activated T cells possess the CD40L (gp 39), important for the subsequent activation of B cells leading to the production of IgE (126) and (127). Addition of anti-CD40 antibody in these cultures, lead to the production of much higher levels of IgE compared to the levels of IgE produced in the absence of anti-CD40. According to Wheeler *et al.*, (1996), anti-CD3 antibody "in vitro", suppresses IgE synthesis (295).

The influence of T cells on the production of IgE depends on the predominate cytokine pattern secreted by these cells. Hence, addition of Th1 clones in a B-cell culture leads to a dose-dependant inhibition of IgE production, mainly due to the presence of IFN-γ

(372). On the contrary, during a predominant Th2 response, seen in allergies and immediate hypersensitivity reactions, T cells will aid the production of IgE, mainly via the secretion of IL-4.

There is now evidence that CD8+ cells can play a role in regulating IgE secretion. Studies by Noble et al., (1995), Croft *et al.*, (1994) and Sad and Mosmann, (1995) have revealed the existence of functionally distinct subpopulations of CD8+ T cells that can be broadly compartmentalised along the same lines as CD4+ T cells, with IL-2 and IFN-γ secretion representing Th1/Tc1 cells, IL-4 and IL-5 secretion representing Th2/Tc2 cells and an unrestricted cytokine profile representing Th0/Tc0 cells (234), (231) and (232). Mingari *et al.*, (1984), showed that certain Tc clones could produce sufficient amounts of IL-4 "in vitro" when stimulated with anti-CD3 or mitogens (224). These cells, now defined as Tc2, are not very cytolitic and are found in abundance in HIV infected individuals and patients with chronic B-cell leukaemia. Maggi *et al.*, (1994), isolated CD8+ circulating T lymphocytes that produced "type 2" cytokines (such as: IL-4, IL-5 and IL-10), with poor cytolitic activity from patients infected with HIV-1 (273). Le Gros and colleagues showed that when CD8+ cells were incubated "in vitro" with IL-4, they became CD4- CD8-, they lost their cytolitic activity and started secreting "type 2" cytokines, such as IL-4 and IL-5 (275). More specifically, Tc2 cells are less effective in killing tumour cells via the Fas-L pathway (373). Stanciu et al., (1996), showed that the secretion of IL-4 by these Tc2 clones can be much higher than the levels of IL-4 secreted by the Th2 cells, and Erard et al., (1993), showed that these Tc2 clones were capable of switching B cells to IgE synthesis (228) and (227).

Interestingly, some Tc2 cells can express the CD40L on their surface which can aid B-cell activation by binding to the CD40 molecule on the surface of mouse B cells, causing

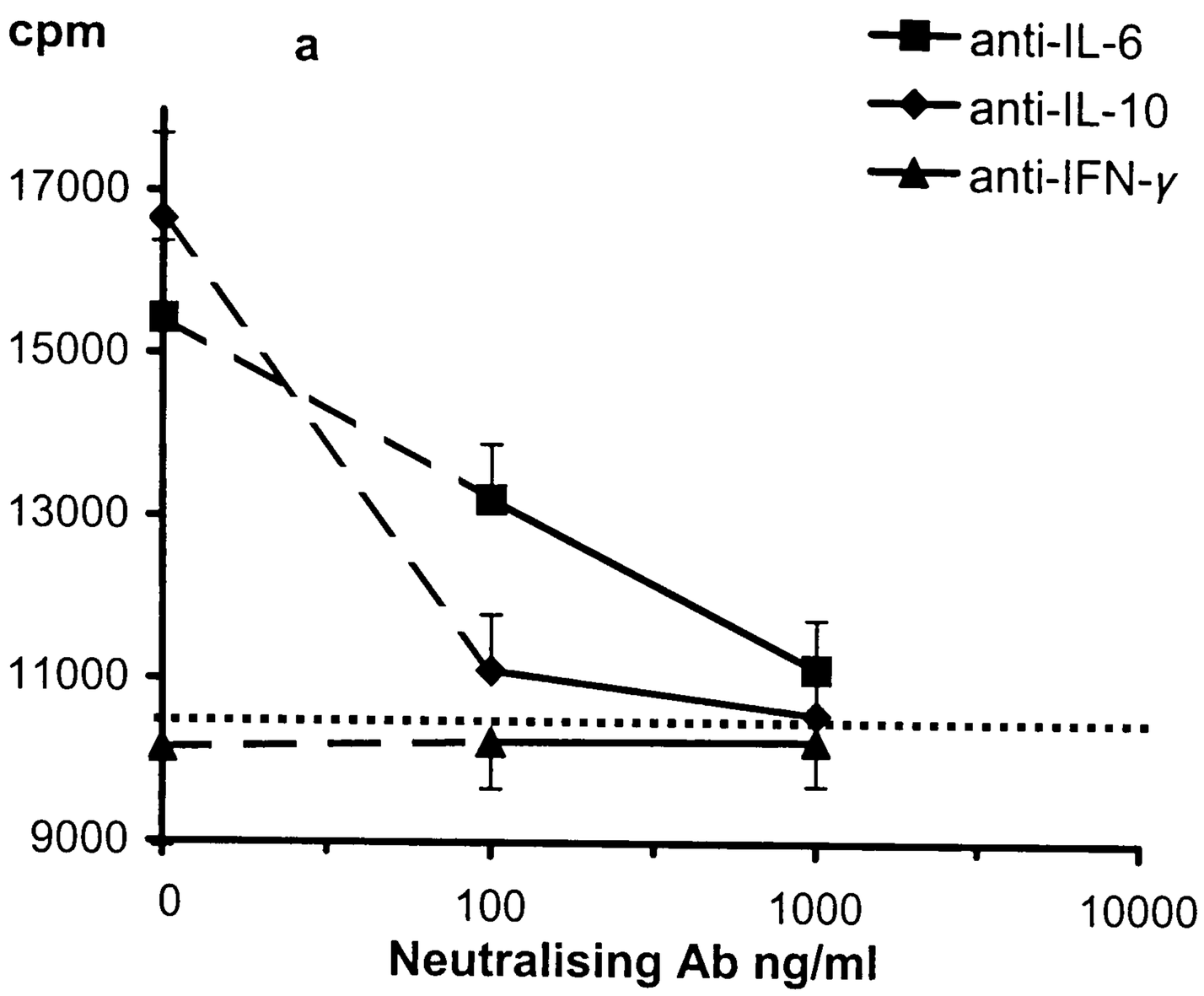
IgE switching (296). Expression of the CD40L was absent from the Tc1 mouse clones, manifesting a further inability of these cells to switch B cells to IgE production.

In this chapter, we confirmed the effect of the various cytokines tested previously on IgE production by using their relevant neutralising antibodies. The cytokine content of the Tc clone supernatants was determined and the effect of these supernatants on IgE synthesis tested in the absence or presence of the appropriate neutralising antibodies.

6.2 RESULTS.

6.2.1 Effect of neutralising antibodies on cytokines controlling B-cell proliferation and IgE production "*in vitro*".

Tonsillar B cells purified by T-cell rosetting were cultured under standard conditions of IL-4 (20 ng/ml) and anti-CD40 (0.5 μg/ml), together with one exogenous cytokine from: IL-6, IL-10, IFN-γ or TGF-β at 100 U/ml, as this concentration, (or greater), has been shown to affect significantly B-cell proliferation and IgE switching "*in vitro*". With each of these cytokines, its appropriate neutralising antibody was added at titrating amounts ranging from: 1-10000 ng/ml, for the azide-free antibodies: anti-IL-4 and anti-TGF-β, while for the azide-containing antibodies (anti-IL-6, anti-IL-10 and anti-IFN-γ), the range was 1-1000 ng/ml. After a 4 day incubation (at 37°C), B-cell proliferation was determined by ^{3}H-Thymidine incorporation. The results showed that both the potentiating effects (of IL-4, IL-6 and IL-10) as well as the suppressive effects (of TGF-β) on B-cell proliferation were largely eliminated at a concentration of 1000 ng/ml or higher of blocking antibody **(Fig.6.1a+6.1b).** Similarly with IgE regulation (after a 10 day incubation), the potentiating effects of IL-4, IL-6 and IL-10, as well as the suppressive effects of IFN-γ and TGF-β, were also neutralised at concentrations of 1000 ng/ml or higher of blocking antibodies **(Fig.6.2a+6.2b).**



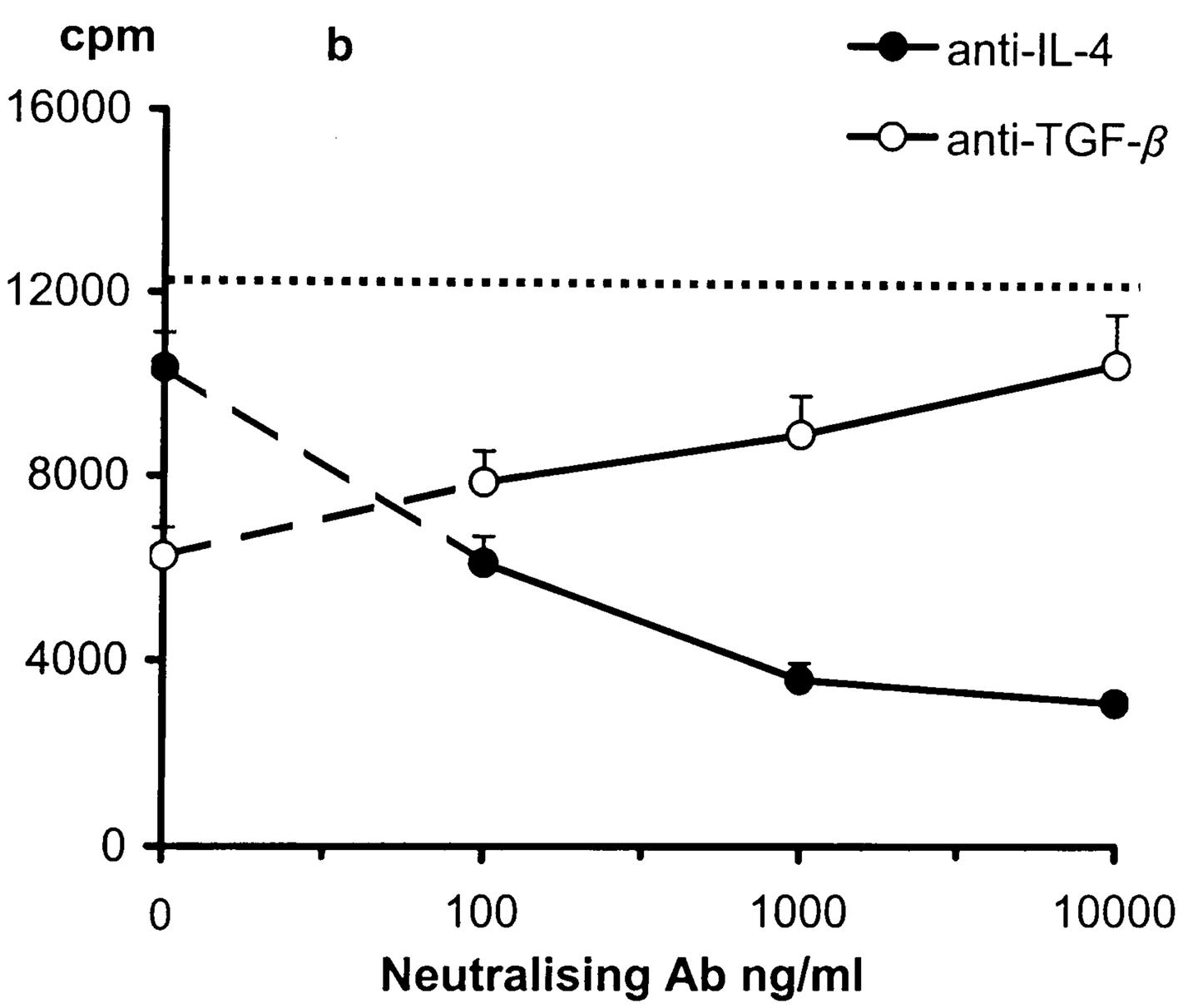
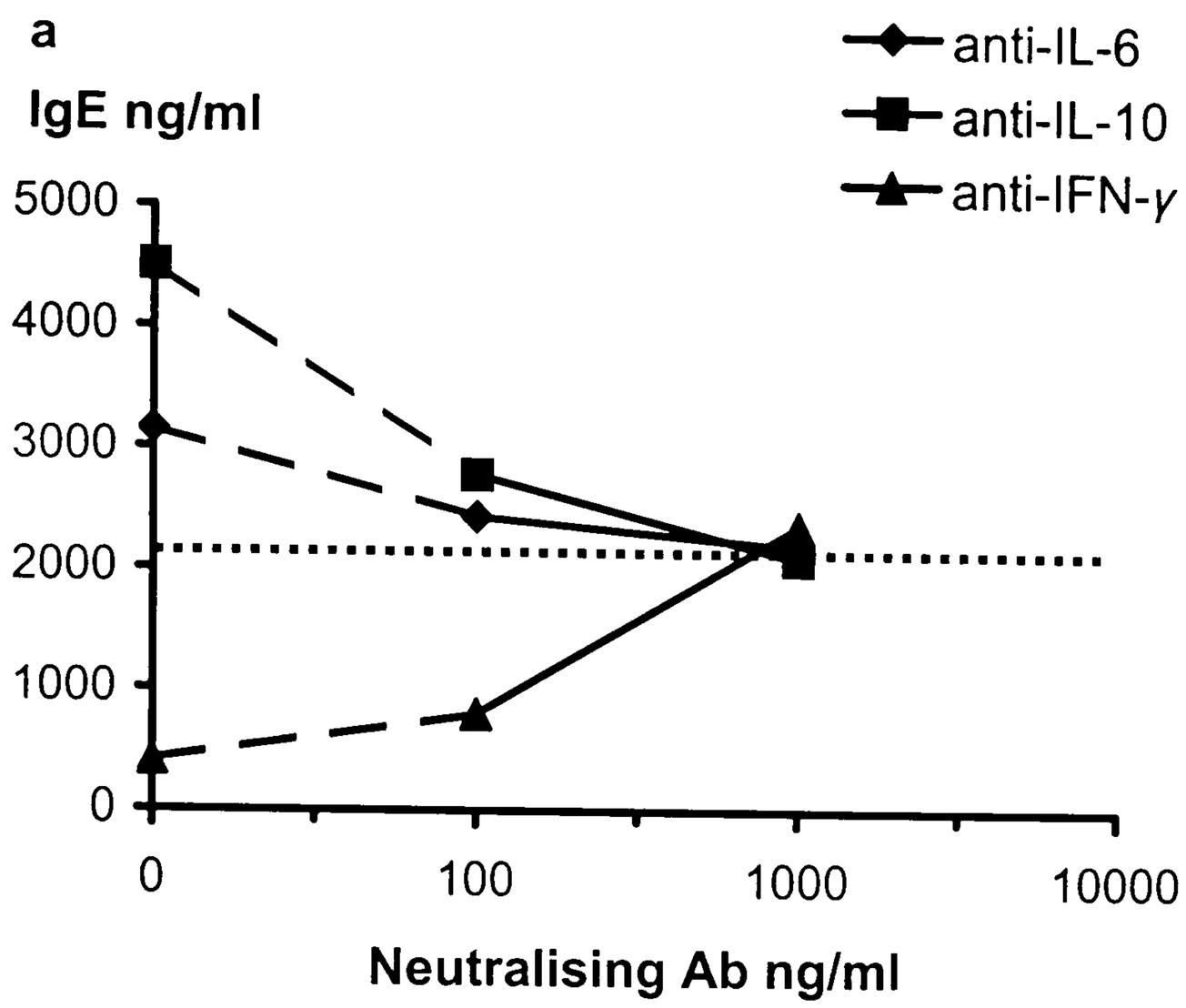


Figure 6.1:Effect of cytokine neutralisisng antibodies on B-cell proliferation. Tonsillar B cells purified by T-cell rosetting were cultured under standard conditions, together with one exogenous cytokine from: IL-6, IL-10, IFN-γ or TGF-β at 100 U/ml. With each of these cytokines, the appropriate neutralising antibody was added at titrating amounts ranging from: 1-1000 ng/ml (anti-IL-6, anti-IL-10 and anti-IFN-γ), or from: 1-10000 ng/ml (anti-IL-4 and anti-TGF-β). After a 4 day incubation, a B-cell proliferation assay was carried out using ^{3}H-Thymidine. The control line shows the B-cell proliferation level in the presence of IL-4 and anti-CD40 alone (standard conditions). These results are representative of three donors. The standard deviations have been calculated (triplicates). Dotted line = Control culture 10,350 cpm.



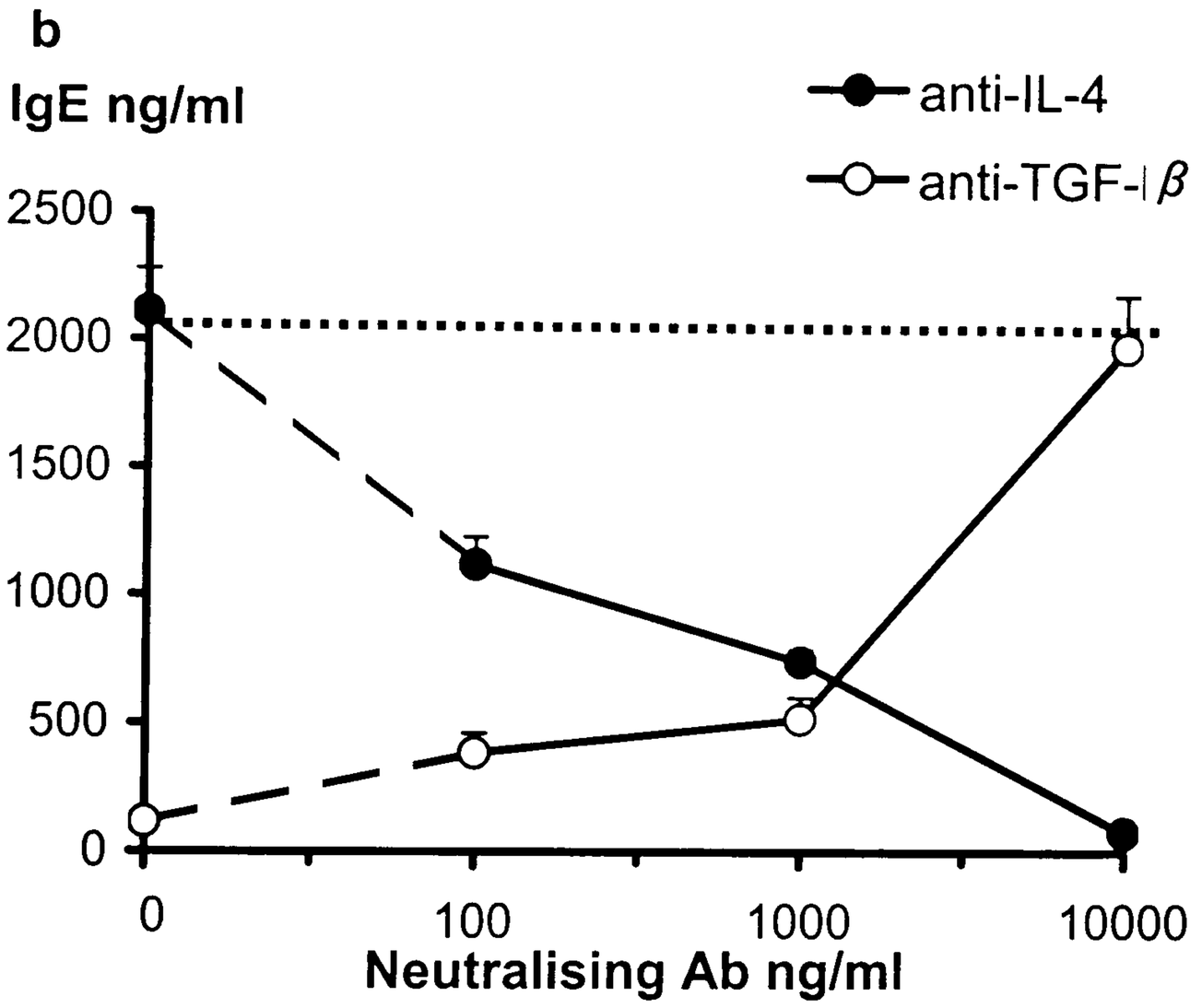


Figure 6.2:Effect of cytokine neutralising antibodies on IgE production. Tonsillar B cells purified by T-cell rosetting were cultured under standard conditions, together with one exogenous cytokine from: IL-6, IL-10, IFN-γ or TGF-β at 100 U/ml. With each of these cytokines, the appropriate neutralising antibody was added at titrating amounts ranging from: 1-1000 ng/ml (anti-IL-6, anti-IL-10 and anti-IFN-γ), or from: 1-10000 ng/ml (anti-IL-4 and anti-TGF-β). After a 10 day incubation, the IgE levels in the supernatants were measured by ELISA. The control line shows the IgE level in the presence of IL-4 and anti-CD40 alone (standard conditions). These results are representative of three donors. The standard deviations have been calculated (triplicates). Dotted line = Control culture 2,110 ng/ml.

6.2.2 Cytokine levels in Tc cell supernatants.

The cytokine levels in the Tc cell supernatants: Clones A111, 1 and 5 (Tc1 type) and B206, 2 and 4 (Tc2 type) were measured by ELISA as described in the materials and methods section. The results were presented as relative amounts for clones A111 and B206 **(fig. 6.3a+6.4a),** and in a quantitative form for clones 2 and 5 (Tc1) and 1 and 4 (Tc2), **(Table 6.1).** It was concluded that clones: A111, 2 and 5 are Tc type 1 due to their high levels of IFN-γ and low IL-4, while clones: B206, 1 and 4 are clearly Tc type 2 clones with high levels of IL-4 and IL-10 (as well as IL-6) and really low levels of IFN-γ.

6.2.3 Effect of Tc clone supernatants on the IL-4-dependent IgE secretion by human B cells *"in vitro"*.

The Tc clone supernatants used were characterised as Tc1 or Tc2 according to their cytokine profiles detected by the cytokine ELISAs **(Fig.6.3a and 6.4a and Table 6.1)**. The supernatants, kindly provided by Dr. Milica Vukmanovic-Stejic, included: the Tc1 clones: A111, 2 and 5 and the Tc2 clones: B206, 1 and 4.

Tonsilar B cells purified by T-cell rosetting, were cultured with IL-4 (20 ng/ml) and anti-CD40 (0.5μg/ml), in the presence or absence of one supernatant at a time. In the case of the supernatants from clones A111 and B206, a titration protocol was carried out which created the following dilutions of the supernatant: 1/2, 1/10 and 1/100. After a 10 day incubation, the B-cell supernatants were collected and their IgE content was measured using an IgE sandwich ELISA. It was shown that the levels of IgE in the cultures increased with increasing concentration of the Tc2 clone supernatant: B206 **(figure 6.4b)**, while the reverse occurred with the Tc1 clone supernatant: A111 **(figure**

6.3b). In both cases, the IgE levels were compared to the control levels where the B cells were cultured with IL-4 (20ng/ml) and anti-CD40 (0.5 μg/ml).

In order to assess the effects of cytokines present in the CD8+ T-cell supernatants, neutralising antibodies were individually added in the cultures. Consequently, anti-IFN-γ was added at the optimum concentration of 1000 ng/ml together with the Tc 1 clones: 2 and 5. Similarly, anti-IL-4 (10000ng/ml), anti-IL-6 and anti-IL-10 were individually added at 1000 ng/ml, together with the Tc 2 clones: 1 and 4. In order to investigate whether the IL-4 content of each of the supernatant was sufficient to switch B cells to IgE secretion, the latter were added in the cultures in the presence or absence of any exogenous IL-4. Further addition of anti-IL-4 (at 10000 ng/ml) with the Tc type 2 cell supernatants would confirm the importance of IL-4 in IgE synthesis. The experiment was carried out twice in triplicates and the 96-well plates were cultured for 12 days. The levels of IgE in the cultures were measured by ELISA. Tc type 1 supernatants: 2 and 5 were able to downregulate the IL-4-dependent IgE switching observed in the control cultures (standard conditions of IL-4 and anti-CD40). In the absence of IL-4, the levels of IgE were negligible. This downregulation was blocked by the addition of anti-IFN-γ antibodies, highlighting the suppressive effect of IFN-γ on IgE production (figures 6.5 and 6.6).

Tc2 cell supernatants: 2 and 4, were able to enhance the IL-4-dependent IgE secretion and were also able to switch B cells to IgE in the absence of exogenous IL-4. Addition of anti-IL-4 suppressed entirely the levels of IgE in the supernatants. When anti-IL-6 and anti-IL-10 were added separately with Tc2 supernatants, reduced the levels of IgE secretion to approximately 75% **(Fig.6.7+6.8).**

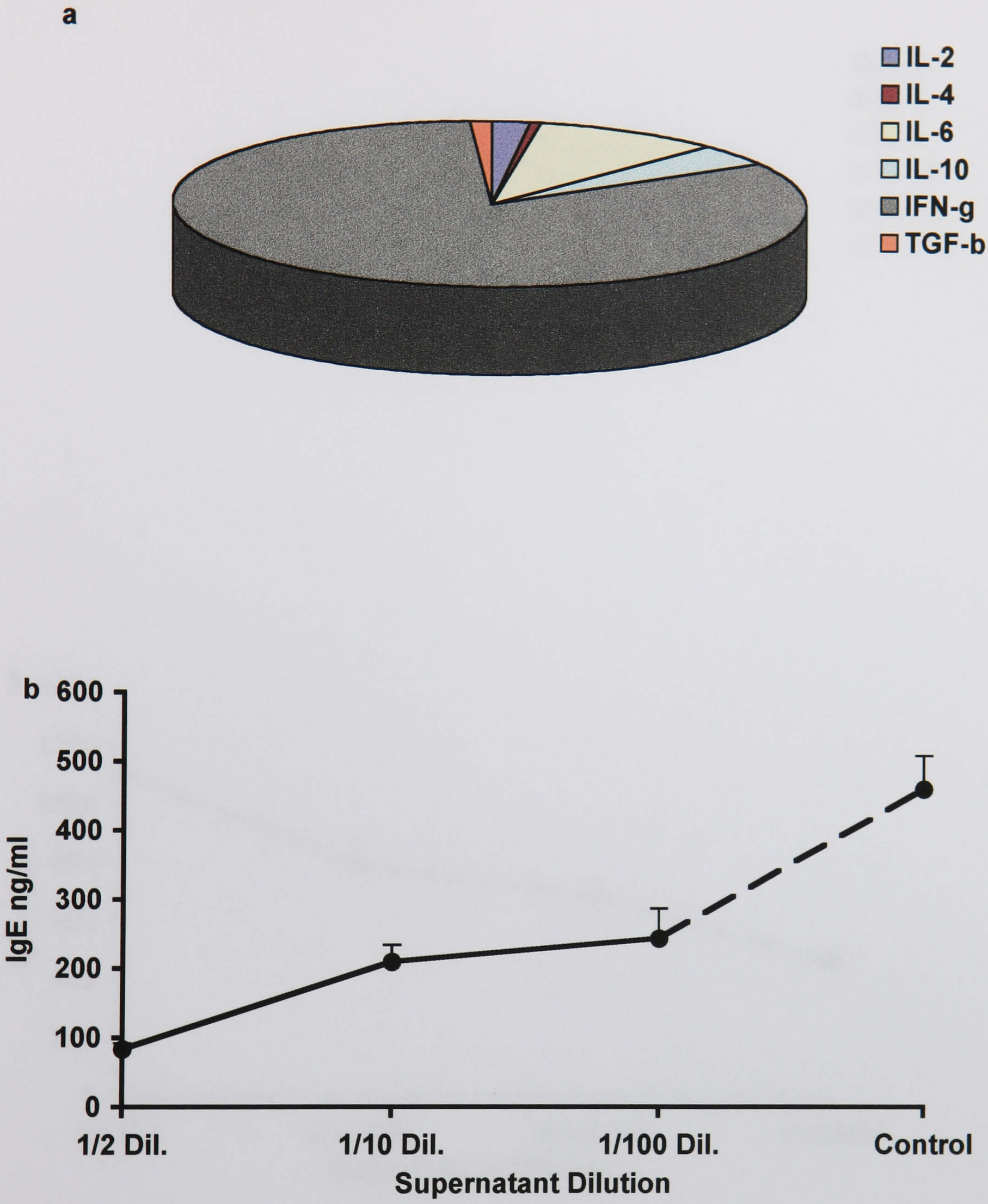
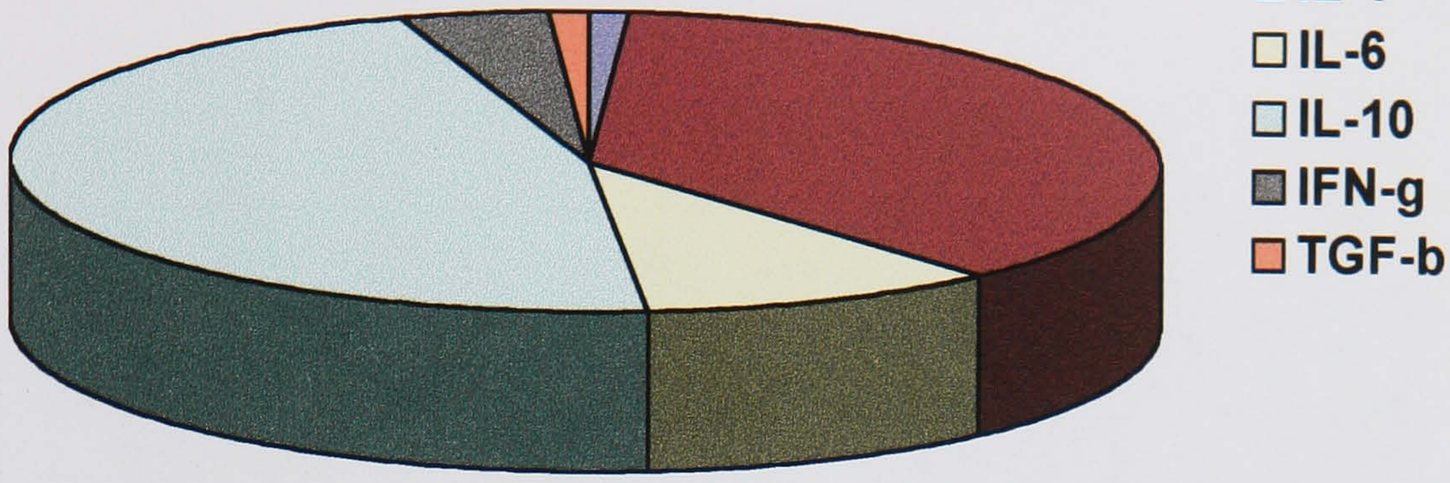


Figure 6.3a: Relative cytokine amounts in A111 clone supernatant.
Figure 6.3b: Effect of A111 supernatant on IgE production.
Tonsillar B cells were purified by T-cell rosetting and put into culture with IL-4 (20 ng/ml) and anti-CD40 (0.5 μg/ml) (Control Conditions). Clone A111 supernatant, (relative cytokine amounts calculated by cytokine ELISAs, presented in figure 6.3a), was titrated in the B-cell cultures at the following dilutions: 1/2, 1/10, 1/100. After 10 days of culture, B-cell supernatants were collected and the IgE levels were measured by ELISA. Results are representative of two donors. Standard deviations have been calculated (triplicates).

a



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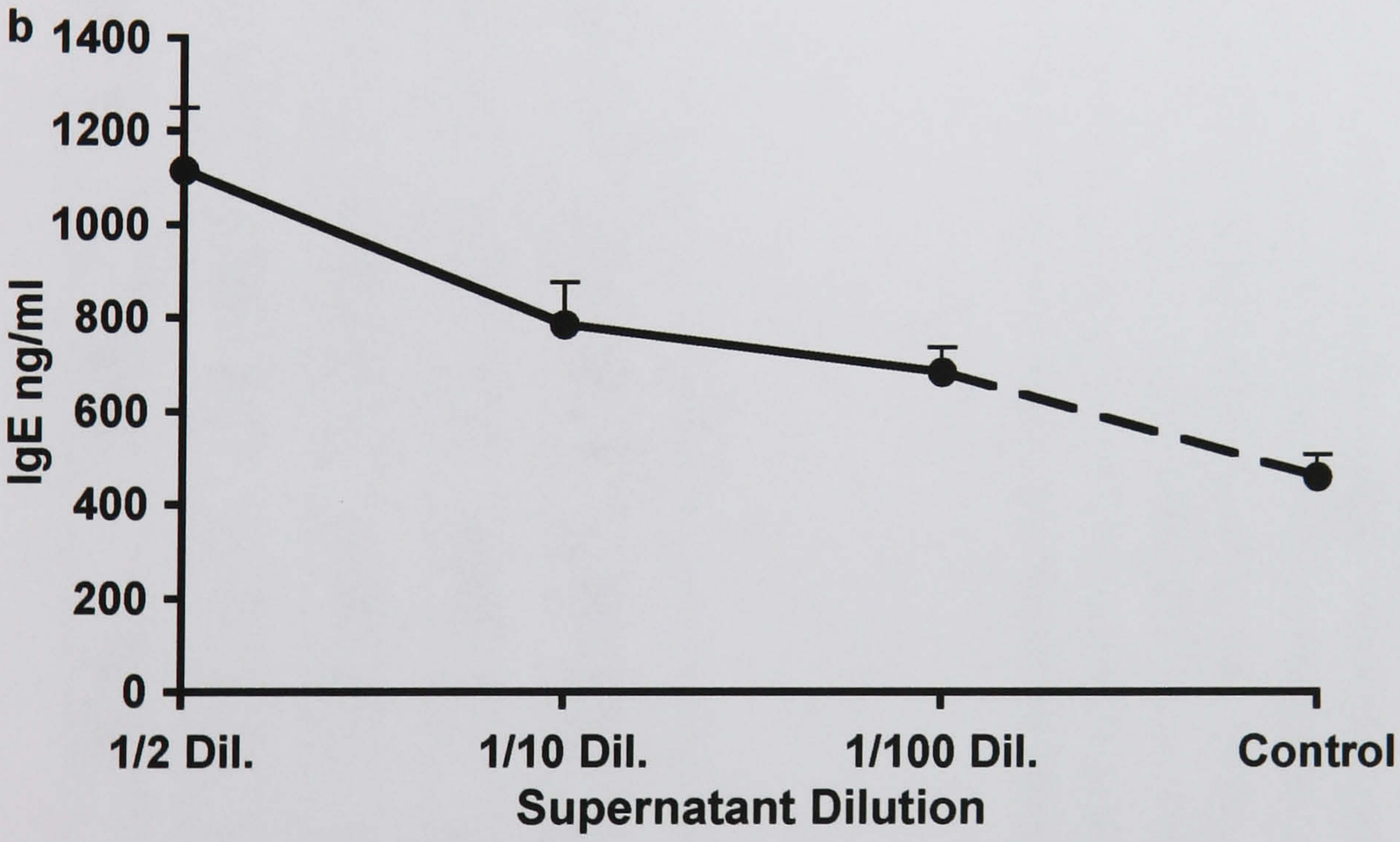

Figure 6.4a: Relative cytokine amounts in B206 clone supernatant.

Figure 6.4b: Effect of B206 supernatant on IgE production.

Tonsillar B cells were purified by T-cell rosetting and put into culture with IL-4 (20 ng/ml) and anti-CD40 (0.5 μg/ml) (Control Conditions). Clone B206 supernatant, (relative cytokine amounts calculated by cytokine ELISAs, presented in figure 6.4a), was titrated in the B-cell cultures at the following dilutions: 1/2, 1/10, 1/100. After 10 days of culture, B-cell supernatants were collected and the IgE levels were measured by ELISA. Results are representative of two donors. Standard deviations have been calculated (triplicates).

CD8+ clones	IL-2	IL-4	IL-6	IL-10	IFN-γ	TGF-β
Tc1 Clone 2	0.4 ng/ml	0.1 ng/ml	1.8 ng/ml	0.8 ng/ml	16.1 ng/ml	0.2 ng/ml
Tc1 Clone 5	0.2 ng/ml	0.9 ng/ml	1.3 ng/ml	1.0 ng/ml	25.3 ng/ml	0.5 ng/ml
Tc2 Clone 1	0.2 ng/ml	7.8 ng/ml	3.0 ng/ml	4.8 ng/ml	0.8 ng/ml	0.3 ng/ml
Tc2 Clone 4	0.2 ng/ml	6.4 ng/ml	1.8 ng/ml	8.1 ng/ml	0.7 ng/ml	0.2 ng/ml

Table 6.1:Cytokine levels in Tc cell supernatants. The cytokine levels in the Tc cell supernatants were measured by direct sandwich cytokine ELISAs. ELISA plates were coated with the catcher monoclonal antibodies (primary) (1μg/ml) and left overnight. Plates were blocked with a blocking buffer for 30 min, the standards and the supernatants tested were added. The top standard concentration used was: 20 ng/ml. Recombinant human cytokines were used as a quality control. After a 2h incubation at ambient temperature the biotinylated secondary antibodies were added (1μg/ml) followed by streptavidin alkaline phosphatase (1:1000 dilution). After a further incubation for 1 h. at ambient temperature, the substrate solution was added (1 p-nitrophenylphosphatase tablet/ 5 ml diethanolamine buffer) and the colour was left to develop for at least 30 min in the dark. The OD values were measured by the Macintosh programme: SOFTMAX.

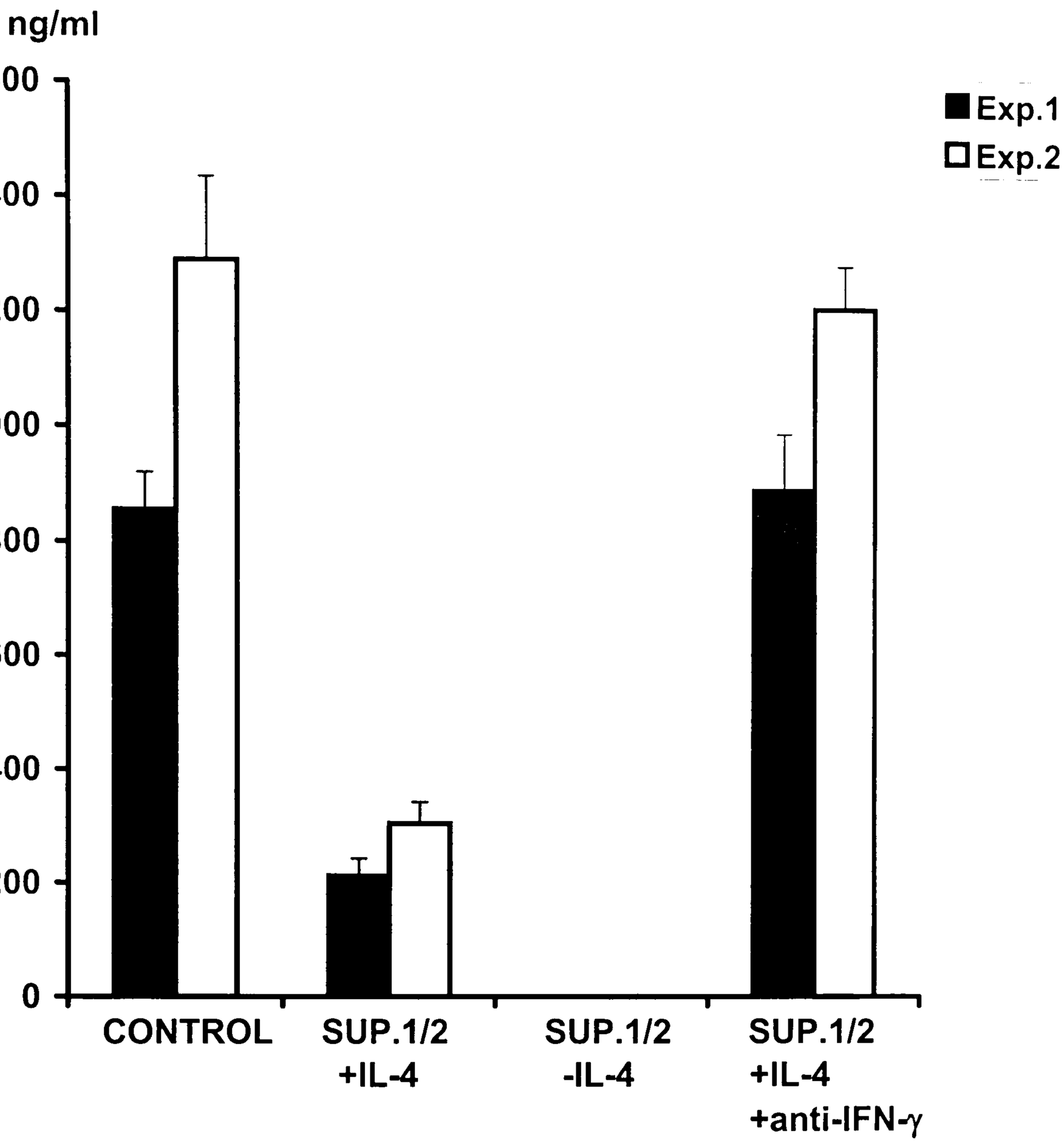

Figure 6.5: Effect of Tc1 clone 2 supernatant on IgE secretion.
Tonsillar B cells purified by T-cell rosetting were cultured under standard conditions of IL-4 and anti-CD40 (control) or in the presence of Tc1 clone 2 supernatant (100µl-1/2 dilution). The supernatant was also added in the absence of any exogenous IL-4. The B cells were cultured for 10 days and their IgE content was measured by ELISA. The experiment were carried out twice (two donors) and the standard deviations were calculated for each donor (triplicates).

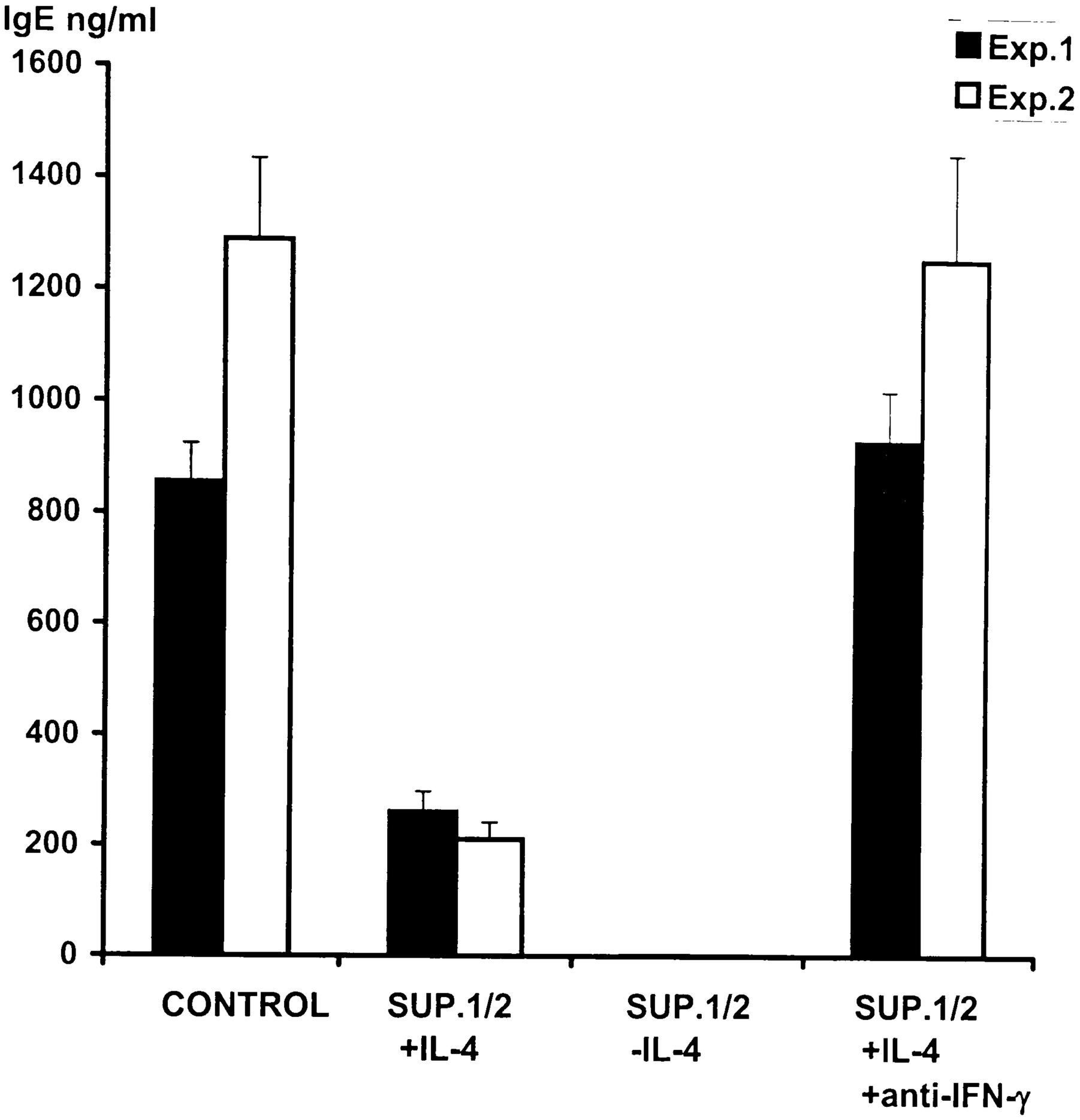

Figure 6.6: Effect of Tc1 clone 5 supernatant on IgE secretion.
Tonsillar B cells purified by T-cell rosetting were cultured under standard conditions of IL-4 and anti-CD40 (control) or in the presence of Tc1 clone 5 supernatant (100 µl-1/2 dilution). The supernatant was also added in the absence of any exogenous IL-4. The B cells were cultured for 10 days and their IgE content was measured by ELISA. The experiment were carried out twice (two donors) and the standard deviations were calculated for each donor (triplicates).

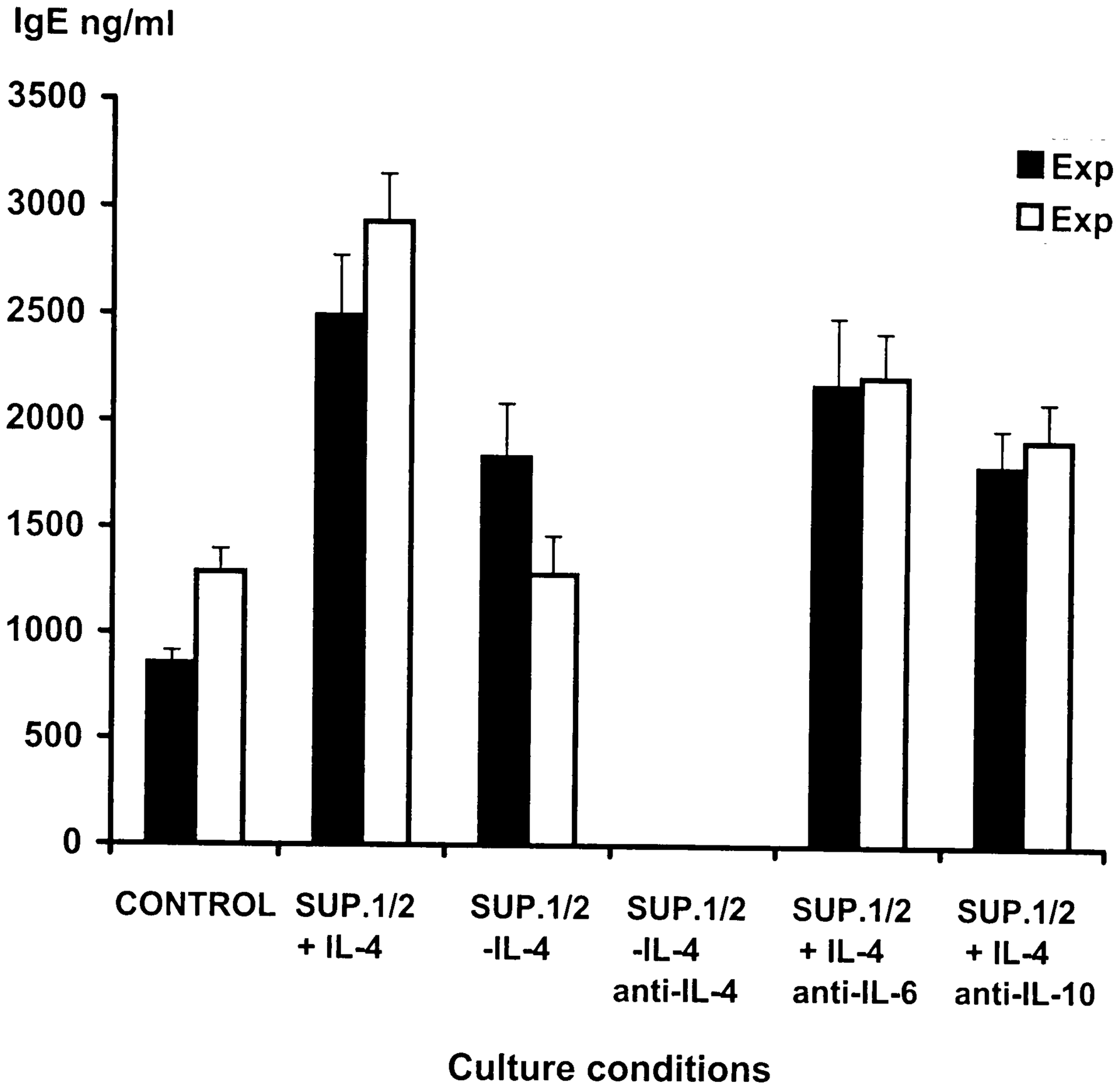

Figure 6.7: Effect of Tc2 clone 1 supernatant on IgE production.

Tonsillar B cells purified by T-cell rosetting were cultured under standard conditions of IL-4 and anti-CD40 (control) or in the presence of Tc2 clone 1 cell supernatant at 100 μl (1/2 dilution). The supernatant was also added in the absence of any exogenous IL-4 ± anti-IL-4 neutralising antibody (1000 ng/ml). Anti-IL-6 and anti-IL-10 neutralising antibodies were also added individually with the Tc2 cell supernatant at standard conditions. The experiment was carried out twice and the standard deviations were calculated.

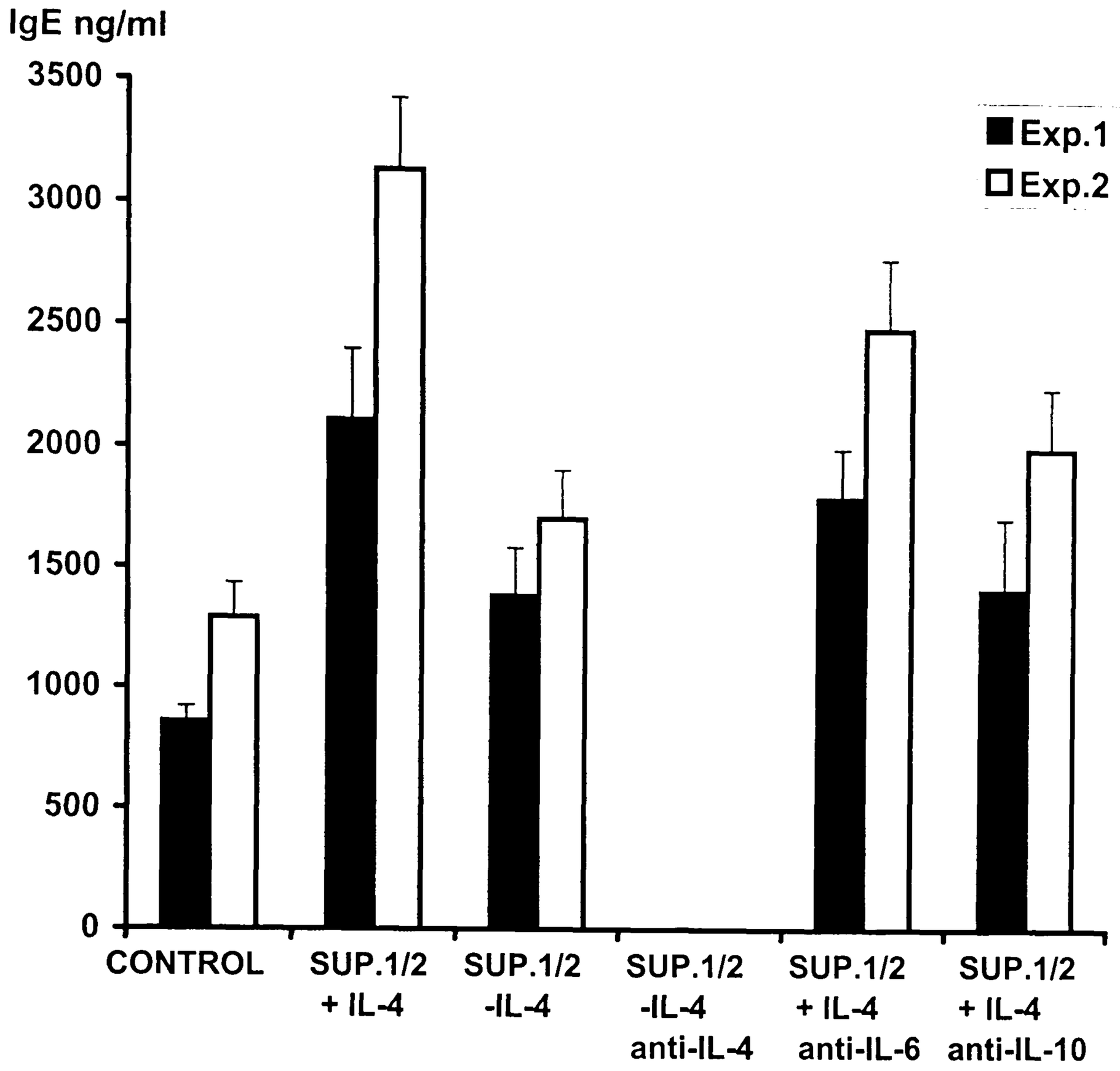

Figure 6.8: Effect of Tc2 clone 4 supernatant on IgE production. Tonsillar B cells purified by T-cell rosetting were cultured under standard conditions of IL-4 and anti-CD40 (control) or in the presence of Tc2 clone 4 cell supernatant at 100 µl (1/2 dilution). The supernatant was also added in the absence of any exogenous IL-4 ± anti-IL-4 neutralising antibody (1000 ng/ml). Anti-IL-6 and anti-IL-10 neutralising antibodies were also added individually with the Tc2 cell supernatant at standard conditions. The experiment was carried out twice and the standard deviations were calculated.

6.3 DISCUSSION.

The possibility that CD8+ cells might be involved directly and autonomously in atopic conditions. has been studied by various groups. For example. Arai *et al.*, (1990). demonstrated that IL-4 producing CD8+ cells could enhance the production of IgE by B cells (374). They also showed that CD8+ Tc2 cells could stimulate mast cell growth and activation. Coyle *et al.*, (1995). showed that IL-5 producing CD8+ cells may cause eosinophilic inflammation following viral infection in allergic mouse models (375). Finally, CD8+ cells are capable of eliciting DTH reactions to viral antigens (376).

CD8+ T cells in HIV-infected individuals that were able to secrete significant amounts of IL-4. an observation which is probabaly related to the elevated IgE levels in these patients (hyper-IgE). These CD8+ cells also expressed high levels of the CD40L on their surface, essential for efficient B-cell activation and immunoglobulin class switching (377) and (277).

In this chapter, we focused on the effects of various Tc cell supernatants (both Tc1 and Tc2 type) on IgE production by human B cells "*in vitro*". Classification of the Tc clones was confirmed by cytokine content of the cell culture supernatants. The results showed a clear cut classification of the clones used. as Tc1 or Tc2. Consequently. clones: A111, 2 and 5 were confirmed as Tc1, having a type 1 cytokine profile in their supernatants characterised by high levels of IFN-γ and much lower levels of IL-4, while clones: B206. 1 and 4 were classified as Tc2, containing high levels of IL-4. IL-6 and IL-10 but lacking in IFN-γ. In both types of supernatants the levels of IL-2 and TGF-β were negligible, thus not considered.

In order to isolate the effects of the individual cytokines in the supernatants on IgE synthesis, their respective neutralising antibodies were used at their optimum concentrations. The latter were established after a series of titration experiments which also confirmed the B-cell proliferative and IgE secreting ability of the corresponding cytokines. The potentiating effects of IL-4, IL-6 and IL-10 on IgE switching and B-cell proliferation were neutralised by the addition of their respective neutralising antibodies. In the case of IFN-γ and TGF-β, their suppressive effect on IgE secretion could be neutralised by the addition of anti-IFN-γ and anti-TGF-β respectively.

The effect of the supernatants used on IgE switching depended on their cytokine content, hence their Tc type. Thus, clone supernatant: A111 could downregulate IgE production by the B cells due to its high levels of IFN-γ while the levels of IgE in the B-cell cultures increased with increasing amounts of the clone supernatant: B206. The effect of the latter was probably due to the high levels of IL-4, IL-6 and IL-10 in this supernatant, as well as the low levels of IFN-γ.

In this study, the Tc2 clones supernatants used (Clones: 1 and 4) were able to potentiate IL-4-dependent IgE production. They could also stimulate B cells to IgE secretion in the absence of any exogenous IL-4, indicating that the content of IL-4 in these Tc cell supernatants was sufficient for this process. The latter effect was neutralised by the addition of anti-IL-4 monoclonal antibodies. The potentiating effects of IL-6 and IL-10 present in these Tc type 2 cell supernatants could be individually blocked by their respective neutralising antibodies.

The Tc type 1 clones used (Clones: 2 and 5), suppressed the IL-4-dependent IgE secretion. This suppression was completely neutralised by the addition of anti-IFN-γ

monoclonal antibodies, indicating that the inhibitory factor produced by these cells was IFN-γ. Tc type 1 cell supernatants were unable to switch B cells to IgE secretion in the absence of exogenous IL-4.

CHAPTER 7: DISCUSSION.

7.1 Cytokine controlled Ig class switching.

In this study, the source of human B cells was freshly isolated tonsils from patients aged between 3-35 years of age. The B cells were purified using two different techniques : T-cell rosetting (negative selection) and CD19+ dynabeads (positive selection). The % of CD19+ B cells obtained by positive selection was similar to that obtained by T-cell resetting, although in the latter method there may be some T-cell contamination (CD3+ / CD19- cells). Addition of autologous T cells showed that the levels of impurities present in the B cell cultures would not affect B-cell proliferation or IgE secretion. Tonsillar B cells consist of naïve B cells, expressing surface IgM and IgD, and isotype committed B cells that have undergone isotype switch and express (or secrete) surface IgG, IgA or IgE but have lost expression of IgM and IgD (313).

Proliferation of B cells purified by CD19+ dynabeads was slightly raised compared to the proliferation of T-cell rosetted B cells, probably due to activation by the CD19 dynabeads By contrast, levels of IgE secreted by the positively selected B cells were lower compared to those isolated by T-cell rosetting, partly due to the lack of T-cell contamination but mainly due to the toxicity of the dynabeads. For this reason, T-cell rosetting was the method of choice.

In order for B cells to switch they need two signals: The first is generated by the engagement of the IL-4R on the B cells by IL-4, while the second signal is driven by the engagement of the CD40 surface molecule by the CD40L on activated T-cells (129), (134) and (378). The two signals have been shown to activate the C epsilon promoter in B-cell lines (378).

The importance of CD40 cross-linking was shown in this study as in the absence of CD40/anti-CD40 interaction. B cells did not proliferate or switch to any immunoglobulin subclass.

Demonstration of the critical role of CD40/CD40L interaction "in vivo" arose from the discovery that the hyper-IgM syndrome, an X-linked immunodeficiency, is due to a genetic alteration of the CD40L gene (379). This disease is characterised by severe impairement of T-cell dependent antibody responses with no B-cell memory, deficient induction of somatic mutations and little or no circulating IgG, IgA or IgE (309).

The importance of IL-4 in IgE secretion was continued in this thesis. In the absence of recombinant IL-4, B cells failed to proliferate excessivelly or secrete any IgE in the cultures. Neutralisation of IL-4 in Tc2 type T-cell supernatants by anti-IL-4 monoclonal antibody blocked IgE secretion by the human B cells "in vitro" (discussed later on). IL-4 (together with IL-13), are the only known cytokines able to induce IgE synthesis "in vitro" when added in the recombinant form (126), (127), (111) and (380). Anti-IL-4 antibodies preferentially inhibit IgE synthesis without affecting the levels of other immunoglobulin isotypes (381), (382) and (170).

In this study, secretion of IgG1-4 and IgA was also investigated. B cells were cultured with various cytokines in the presence or absence of IL-4. Anti-CD40 was always present in the cultures as it would ensure B cell survival and provide signal 2 for the switching process. IL-10, IL-12 and IFN-γ were able to increase the secretion of IgG1 but only IFN-γ though could produce the same effect in a dose-dependent fashion in the absence of IL-4. This indicates that IFN-γ has the ability to act directly on the human B cells and cause them to produce IgG1 confirming earlier work by (298), (383) and (384).

It is likely that the effect of IL-10 was due to its immunoproliferative properties in the presence of IL-4. The proliferative activity of IL-10 has been demonstrated (350). The ability of IL-10 to switch anti-CD40 activated tonsillar B cells in the absence of IL-4 has been also reported (313). Nonoyama et al., (1994) suggested that IL-10 was an IgG2 switch factor for peripheral B cells (385), while Ehlers and Smith (1991), showed that IFN-γ plays an important role in the IgG2 switching of human neonatal B cells (386). These studies provide the only evidence in the field of IgG2 class switching. In our study, regulation of IgG2 production remained unclear. The general switching ability of IL-10 towards most IgG subclasses was also shown by Malisan et al., (1996), by proving that the latter cytokine promoted switching recombination to Cγ genes in CD40-activated human tonsillar naïve B cells (387). In our study, no cytokines were able to potentiate the secretion of this immunoglobulin, even in the presence of IL-4, suggesting that there were probably no IgG2 committed B cells in the tonsils of the donors used in these experiments. IgG2 is normally directed against carbohydrate antigens which usually are T-cell independent, which means that they might also be cytokine independent.

The enhancement of IgG1 by IL-12 was surprising as the latter cytokine inhibits the IL-4-dependent proliferation of the B cells. It seems more likely that its action was indirect via the production of IFN-γ by the B cells. This explanation though is not heavily supported because if IL-12 was acting through the induction of IFN-γ, it should be able to switch B cells to IgG1 production even in the absence of IL-4. Perhaps IFN-γ was not around long enough or was at too low a concentration. Furthermore, once B cells have been stimulated for a number of days it may be hard to induce novel switching.

Interestingly, Kawano et al., (1995), showed that IgG1 switching in human B cells can be upregulated by IL-6 provided that the cells have been previously activated by antigen such as pokeweed mitogen (PWM) (388). In our study, IL-6 did not seem to have an effect to any of the immunoglobulin subclasses tested.

Briere et al., (1994), showed that IL-10 could switch human B cells to IgG3 production (313). In our study, IL-10 could enhance the secretion of IgG3 by the human B cells but probably via clonal expansion of rather than direct switching, as the potentiating effect of IL-10 was lost in the absence of IL-4, even at the highest concentration tested (1000U/ml). The ability of IL-6 to switch antigen-activated B cells "in vitro" towards IgG3 production was shown by Kawano et al., (1995), but this was not confirmed by our results (388).

IL-6 was able to induce IgG4 production but only in the presence of IL-4 suggesting clonal expansion of pre-committed B cells. It's interesting that IL-6 was able to expand the IgG4 committed B cells but not the IgG1 B cells which were shown to be present in the tonsils. IL-4 and IL-10 were found to be true switch factors for IgG4 (389), (153) and (146). The effect of IL-10 was novel as switching to IgG4 by IL-10 has only been observed in PBMC cultures or in purified B cell cultures (313). Interestingly, IL-12, IFN-γ and TGF-β blocked IL-4 driven IgG4 production in human B cells at 100U/ml.

As previusly described, TGF-β was found to enhance IgA secretion by human B cells in an IL-4-independent manner. This has been previously demonstrated (390), (391) and (322). In addition, TGF-β induces the production of germline α mRNA transcripts, a marker of IgA isotype switch (78). Malisa et al., (1996) and Defrance et al., (1992) suggested that IL-10 can be a switch factor for IgA as the latter cytokine may promote

switch recombination to Cα genes (387) and (318). In our study, the IgA potentiating effect of IL-10 was due to B cell expansion. Despite the existing controversy concerning the mechanism of IgA class switching, the critical role played by TGF-β is without a question.

7.2 Effect of cytokines on IgE production.

The importance of IL-4 in providing the second signal in IgE switching (first signal: CD40 ligation) is widely accepted and also confirmed in this study. The effect of cytokines on IgE production were tested in the presence of IL-4, as the only other cytokine found to be able to switch B cells on its own was IL-13. The amount of IgE secreted by IL-13 stimulated B cells was 10-fold less compared to IL-4 driven ones and there was no additive or synergistic effects between the two cytokines. This observation was in accordance with the findings of Punnonen et al., (1997) and de Vries (1998) (140) and (392). IL-13 shares many, but not all, of its biologic activities with IL-4. For example, T cells do not express functional IL-13 receptors. Both cytokines have growth promoting effects on normal B cells activated by anti-IgM or anti-CD40 mAbs and prevent apoptosis of these cells. Although IL-4 and IL-13 both induce IgE synthesis "in vitro", the relative contribution of the two cytokines to IgE production "in vivo" remains to be determined. Such studies are hampered by the absence of suitable animal models because IL-13, in contrast to IL-4, does not act on murine B cells (392).

IL-6 and IL-10 were both found to enhance B cell proliferation and the IL-4-dependent IgE secretion "in vitro". The potentiating effects of IL-6 were first shown by Maggi et al., (1989) and Vercelli *et al.*, (1989), followed by Bjork and Paulie (1993), using both purified B cells (143) and (127) and PBMCs (347). IL-6 activity might have a dual nature as depletion of monocytes in the PBMC cultures decreased the secretion of IgE (127). Hence both an indirect and direct effect of IL-6 on the human B cells is suggested.

In the case of IL-10, the augmenting effects were unexpected. IL-10 would have been expected to have an inhibitory effect on IL-4-dependent IgE production. However the inhibitory activity of IL-10 takes place mainly in PBMC cultures indirectly via T-cells and monocytes. Downregulation of the CD40L on the former cells is the main immunosuppressive mode of action. In addition it's been shown that depletion of monocytes reduces the inhibitory effects of IL-10 on the IL-4-dependent secretion by the human B cells (317), (393) and (153). IL-10 has been found to decrease ε transcript expression and IgE production by IL-4 or IL-13 stimulated PBMCs (183) and (153). Fluckiger et al., (1993), showed that the downregulatory effect of IL-10 was via increased expression of the IL-2R on B cells, hence enhancing the inhibitory role of IL-2 in the IL-4-dependent IgE switching process (394). In our study, both IL-10 and IL-2 enhanced B cell proliferation while IL-2 inhibited IgE secretion. The augmenting effect of IL-2 on B cell proliferation paired with the blocking effect on the IL-4-dependent IgE switching process, was also shown by Romagnani et al., (1986) and Miyajima et al., (1991) (395) and (145). The latter group demonstrated that the suppressor pathway was different to that mediated by IFN-γ. In our study, the dose-dependent decrease in IgE

secretion by IFN-γ, was thought to be due to a direct effect on the molecular mechanisms of Ig class switching as B-cell proliferation was unaffected. It has been suggested that the effect of the latter cytokine on IL-4-dependent IgE switching might have various modes of actions. For example IFN-γ might modulate IgE switching at the molecular level (396). Alternatively it might regulate expression of co-stimulatory molecules on the surface of immune cells. More specifically, in this thesis we found that IFN-γ has a downregulatory effect on the IL-4R on the B cells thereby reducing the potency of IL-4 for B cells, suppressing the IgE production by the already switched cells. Whatever the case might be, IFN-γ has been shown to block IgE secretion by both murine (170) and human purified B cells (397). Comparable effects on IgE class switching by IFN-γ have also been observed in PBMC cultures (109), (110), (398) and (170). Cytokines can block switching of certain immunoglobulin subclasses by preferentially inducing the switching of others (also discussed later on). Thus, since IFN-γ and TGF-β could switch B cells to IgG1 and IgA respectively, it might explain their inhibitory action on IgE synthesis by the human B cells "in vitro". The inhibitory action of TGF-β was also observed by Stavnezer et al., (1995) (337). In our study, IL-5, IL-7 and IL-9 did not have an effect on either B cell proliferation or IL-4-dependent IgE production.

The final cytokine that showed a downregulatory effect on B cell proliferation and IgE secretion was IL-12. The dose-dependent decrease in B cell proliferation could be one mode of action of IL-12 in blocking IgE synthesis "in vitro" although it has been widely suggested (and also shown in this study), that the main inhibitory effects of this cytokine are via the induced production of IFN-γ (399), (400) and (401). This observation could

be confirmed by using anti- IFN-γ and anti-IL-12 neutralising antibodies simultaneously in the B cell cultures.

Recently, the effects of IL-12 on IgE switching have been related to IL-18. Extensive studies have shown a homology between the IL-12 and the IL-18 receptors indicating similar signalling pathways (341). Both receptors have been detected on human naïve B cells and a synergistic effect between the two cytokines at inhibiting IFN-γ secretion has been established (342), (343), (344) and (157). In this study, IL-18 on its own upregulated IL-4-dependent IgE synthesis while addition of IL-12 blocked this enhancement.

The effects of cytokines on B cell proliferation and IgE secretion, tested in this study, were confirmed by the use of their relevant neutralising antibodies. The use of such antibodies at their optimum concentrations re-adjusted proliferation and IgE secretion to control levels.

7.3 Modes of action of cytokines in IgE production "*in vitro*".

Having established the effects of different cytokines on IgE class switching their possible modes of action were investigated. IL-13 was the only cytokine that was able to induce IgE synthesis in the absence of IL-4. The great homology of IL-4 and the IL-13 receptors indicates the sharing of common signalling pathways between the two cytokines. something that might explain the IgE switching ability of IL-13. The

difference of the IgE levels produced by B cells stimulated by IL-4 or IL-13 were probably due to the higher proliferative activity of the former cytokine on B cells.

The potentiating effects of IL-6 and IL-10 were found to be IL-4 dependent and were probably brought about via the expansion of B cells induced to make IgE by the exogenous IL-4 added in the cultures. In the absence of IL-4, neither of the two cytokines were able to stimulate B cells to produce IgE or significant proliferation. The proliferative effect of IL-6 on the human B cells related to the induction of IgE synthesis was also shown by Jeppson et al., (1998) and Bjork et al., (1998) (366) and (346). In the case of IL-10, the potentiating effect towards the IgE secretion process was novel, as IL-10 has been mainly shown to downregulate the process in PBMC cultures. The proliferative effect of IL-10 (in the presence of IL-4) was shown by Nagumo and Agematsu (1998) and Tangye et al., (1998). Jeannin *et al.*, (1998), proved that the time of IL-10 addition in B cell cultures can be significant (350), (402) and (317). The high correlation between B-cell proliferation and IgE secretion for both IL-6 and IL-10 was also shown by the construction of Linear Regression Plots (see chapter 4).

The inhibition of IgE secretion by IL-12 was partly due to the induction of IFN-γ secretion by the B cells in culture. The ability of IL-12 to induce IFN-γ production by murine T cells was shown early on (401), accounting for the downregulatory effects of IL-12 on IgE production. The ability of IL-12 to induce the secretion of IFN-γ by human T cells is established (157). Furthermore, it has been shown that IL-12 can have the same effect on human B cells (343) and (341), a fact which is in agreement with our findings. Gagro and Gordon (1999), suggested that the culture of B cells with IL-12 can

cause a polarisation of the latter towards a type 1 phenotype, which would secrete a Th1 type cytokine profile (369).

Of course the induction of IFN-γ secretion by IL-12 need not be the only mode of action of this cytokine in blocking IgE synthesis, as our results indicated a decrease in B cell proliferation which would not be brought about by IFN-γ as previously shown. This indicates a direct effect of IL-12 on the human B cells resulting in the inhibition of IgE secretion "in vitro".

An alternative way in which cytokines might indirectly block the secretion of a certain immunoglobulin is by preferentially inducing switching towards another isotype. If the switch regions for two specific immunoglobulins lie close in the germline DNA and their promoters have similar base sequences (e.g.: due to overlap), then it is possible that both molecules would be transcribed, translated and eventually secreted by the cell. This is the case between IgE and IgG1 in mice and IgE and IgG4 in humans. In our study preferential switching to IgG1 was induced by IFN-γ. Increasing the concentration of this cytokine increased the levels of IgG1 in the B-cell cultures while the levels of IgE fell in a dose-dependent fashion. Preferential switching is a mode of action for TGF-β regarding the downregulation of IgE synthesis by the human B cells. The role of the latter cytokine in IgA switching in both murine and human B cells is widely accepted. This is in accordance with our results in which IgA secretion was TGF-β dependent. The precise protocol used might also provide a reliable "in vitro" system for high-rate IgA class production, the lack of which has been described as a major obstacle in studying the switching of this immunoglobulin (319). The fact that both IFN-γ and TGF-β can stimulate B cells to make IgG1 and IgA respectively, in the absence of IL-4, defines

them as true immunoglobulin switch factors. In agreement with this statement, Kim and Kagnoff (1990) showed that the effect of TGF-β on switching human B cells to IgA secretion was direct and not via the clonal expansion of pre-committed IgA+ B cells (322). The binding of TGF-β to its receptor initiates a signalling pathway involving the activation of downstream effectors termed Smad proteins (403) and (404). These Smad complexes translocate into the nucleus where they bind specific DNA sequences in target promoters, thereby activating transcription leading to IgA class switching (405). Hence, the downregulatory activity of IFN-γ and TGF-β on IgE secretion, even in the presence of IL-4, was expected. It is worth highlighting the difference in the inhibitory potency of IFN-γ and TGF-β on B cells compared to IL-4. Although the first two cytokines can readily inhibit IgE production, IL-4, a true IgE switch factor, could not inhibit the secretion of neither IgG1 nor IgA. The "near perfect" negative coefficient correlation between B-cell proliferation and IgE production under the effect of TGF-β suggests that the suppressed IgE levels secreted by the B cells is also related to the dose-dependent decrease of their proliferation.

Regulation of the IL-4R could represent a mode of action for certain cytokines that modulated IgE secretion "*in vitro*". Indeed, Keegan (1991), demonstrated increased expression of the IL-4Rα subunit on human B cells (406) while Ikizawa et al., (1995) suggested that the signal transduction mediated by the up-regulated IL-4R on B cells from allergic individuals may be intimately associated with the induction of isotype switching to IgE that leads to mature C epsilon transcription and IgE production (368). This was further supported by Nasert et al., (1995) and Renz (1999), who administrated sIL-4R in PBMC cultures, and significantly blocked IgE production (407) and (408).

Binding of IL-4 to the IL-4R causes activation of STAT6 which is in turn dependent on the presence of .. JAK3 within the receptor complex (409).

In our study. IFN-γ and TGF-β were found capable of downregulating IL-4R expression on the human B cells after 4 days of culture. The result obtained with the former cytokine was in agreement with So et al.. (2000) who proved both on the cellular and the molecular level (presence of mRNA transcripts) (352). The effect of TGF-β was novel although it does not contradict the rest of our results as switching to IgA was IL-4-independent. Interestingly, downregulation of IL-4R by IFN-γ did not affect B cell proliferation which might suggest that the signals required for the latter act early on in the cultures, before day 4, in which case IL-4R expression would be still unaffected.

Downregulation of the IL-4R observed by IL-12 was probably indirect via the induction of IFN-γ secretion by the human B cells. IL-12 induced production of IFN-γ was observed at day 6 of culture while the decreased levels of IL-4R expression occurred at day 8. Our results would suggest a 2-4 day period required for the inhibitory action of IFN-γ on the regulation of IL-4R. IL-2 did not affect IL-4R expression and thus the mode of IgE inhibitory action of this cytokine remains unclear.

It has to be noted that although the downstream events of IL-4R activation consist of multiple signaling cascades, the biologic function of IL-4 may be regulated by the combination of different signaling molecules, depending on the cell types (410).

7.4 The effect of CD8+ Tc1 and Tc2 cell supernatants on IgE secretion "*in vitro*".

Having established the contribution of individual cytokines to IgE secretion "*in vitro*", our aim was to investigate the suppressive effects of Tc1 and the enhancing effects of Tc2 clone supernatants on IgE secretion. For this purpose, CD8+ (Tc) clone supernatants were used containing distinct cytokine profiles. This experimental approach would help us understand the regulatory role of the CD8+ cells on the IgE levels "in vivo".

In our study, the Tc1 clones (2 and 5) were generated with IL-12, while incubation with IL-4 gave rise to the Tc2 type clones (1 and 4) (work by Dr. Milica Vukmanovic-Stejic). This technique was established by Byun et al., (1994) (240). T cells were stimulated with anti-CD3 and anti-CD28 rather than PMA and ionomycin, so that activation signals would not be passed on to the B cell cultures and bias our results.

The cytokine profiles in the Tc clone supernatants confirmed the nature of the clones with the Tc1 clones containing high levels of IFN-γ and the Tc2 clones having high amounts of IL-4, IL6 and IL-10. IL-2 and TGF-β levels were negligible in both Tc supernatants.

The effects of the Tc supernatants on IgE synthesis "in vitro" were confirmed by the use of neutralising antibodies. Consequently, the downregulation in IgE switching induced by the Tc1 clones: 2 and 5 was found to be due to the high levels of IFN-γ as their ability to inhibit IgE production was neutralised by the anti- IFN-γ antibody. Unsurprisingly, Tc1 clone supernatants were unable to switch human B cells to IgE synthesis in the absence of exogenous IL-4 as the latter cytokine was also absent from these supernatants. By contrast, the Tc2 clone supernatants: 1 and 4, were able to induce IgE

production in the absence of any exogenous IL-4 as they contained sufficient amounts of this cytokine. Similar results were observed with the Tc1 clone supernatant: A111 and the Tc2 clone supernatant: B206. The enhancement of IgE secretion observed by the addition of the Tc2 clone supernatants was confirmed by addition of anti-IL-4 neutralising antibody which completely blocked the secretion of this immunoglobulin. IgE synthesis by the human B cells cultured with the Tc2 clone supernatants were also due to the presence of IL-6 and IL-10 secreted by these clones.

7.5 FURTHER WORK.

The advantage of the experimental approach in this study was that by working at the protein level we were measuring the amounts of IgE actually being produced by the human B cells, which has a higher physiological significance compared to the DNA / RNA level. The drawback though was that we were assessing IgE production rather than IgE switching. It would be interesting to assess the effects of cytokines and CD8+ T cell supernatants on actual IgE switching. This could be done by two ways: Either via the use of naïve B cells (IgM+/IgD+/IgE-), hence any IgE measured would be due to actual switching, or by detecting ε mRNA transcripts by RT-PCR. A combination of the two methods would provide further accuracy and specificity in the study of IgE switching.

Search for the true switch factors of IgG2 and IgG3 in a similar way would be interesting.

Investigate the existence of B1 / B2 cell populations and account for their cytokine profiles both on the molecular (RT-PCR) and the protein level (ELISA). How could these cells affect IgE switching via soluble molecules or even specific surface markers.

It would also be interesting to study the ability of Tc2 cells in IgE switching "in vivo".

Setting up co-cultures of Tc1 and Tc2 clones with autologous B cells could be also another interesting approach. Assessment of CD8+ killing vs. stimulation (i.e. IgE switching) could be carried out giving possible answers on the role of Tc (CD8+) cells "in vivo".

Similarly, co-cultures could be set up between B cells and other autologous professional antigen presenting cells (APC), such as dendritic cells and macrophages, and study their effect on IgE class switching. The effect of various cell surface markers on the APCs could be assessed.

It would be also interesting for our study to use trimeric anti-CD40 (now commercially available). A possible stronger induction of IgE switching is expected which may offset, partly or totally, the inhibitory effects of factors such as IL-12 and IFN-γ.

Study of the IL-4R regulation at the molecular level is also compelling. Detection of IL-4Rα and γ chain mRNAs, by RT-PCR would be one approach. This method could be used to confirm the effect of cytokines on IL-4R expression "*in vitro*". Similarly, test the

effects of Tc clone supernatants on the expression of the IL-4R. A decrease in the levels of expression of the receptor would be expected by Tc1 type supernatants.

To take it further, a relationship should be established between STAT 6 and IL-4R expression. It would be of interest to study whether the two molecules are mutually dependent and whether factors regulating IL-4R expression affect the STAT 6 signalling molecule.

Finally, studying the effects of various factors on the regulation of IFN-γ and TGF-β receptors may provide us with a greater insight on the regulation of IgE switching.

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